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**VOLUME-15 NUMBER-3 (JULY-AUG-SEP 2022)**

## REVIEWS AND CASE REPORTS



## RESEARCH ARTICLES

# Bioscience Biotechnology Research Communications

Open Access International Journal

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**Editors Communique**

Have we tamed the coronavirus? May be yes,
as pandemics do not die, they can only be faded !

Science and technology has made it possible, in the shortest span of time, it has shown that with firm determination and international cooperation, we can win over the onslaughts of even the worst of the pandemics. COVID-19 is perhaps fading over now, due to our coordinated efforts worldwide. Though we have lost millions, in the two year period, partly due to the mishandling of the viral attacks and somewhat by our own follies and carelessness. Anyway lessons learnt from the past, always make us more stronger and determined. Let us now not relax and work on a better mode, as all is still not well yet. The almost taming of the virus and its cousins have indicated some of the concealed failures, on which we have to focus now. We have to be more vigilant, and even a bit of laxity can spoil the good work done. On societal and governmental parts, utmost care and caution is required on a long term basis.

On behalf of Bioscience Biotechnology Research Communications, we falter at words to express our deep sense of solitude and grief on the catastrophic events of the world wide pandemic, spanning over two years now. We pray for the strength to bear this universal calamity and come up with long lasting fortitude to eradicate it soon.

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Quality publication is one of the ways to keep science alive, and good journals have a leading role to play in shaping science for humanity! As teachers, we have great responsibilities, we have to advocate our students to accomplish and show them the path to test their mettle in hard times to excel, especially in the post COVID 19 era. Science and its advocates will rise more to the occasion and will soon provide succor to the already grief stricken humanity.

Sharique A. Ali, PhD
Editor-in-Chief

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# Clinical Trial Phases and their Registration in India: A Systematic Review

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## ABSTRACT

The rationale behind writing this review article is to give an introduction to the clinical trial, its phases and the current scenario in clinical research in India. This article gives a brief idea about the phases of the clinical trial. The reader can find the process of trial in step by step manner with the government regulatory body that has a major role to ensure the safety of the subject involved in the experimental study, with appropriate protocol and approval of the whole experimental study. This paper describes the role of the ethics committee, the investigator and sponsors' responsibilities along with DCGI workflow, regulations of study. This study also discuss what is CDSCO, ICH-GCP government body in clinical trials etc. The clinical trial has a crucial role in serving good health to the public and developing new promising drug candidates in the treatment of diseases. The new drug candidate and therapy enhance the quality and lifespan of the patient. Nowadays the number of clinical trials has increased in biomedical research so there is a huge need to make transparency and easy accessibility of trial studies to general people. Hence the development of CTRI has been done. The regulatory authority strictly observes whether the guidelines are properly followed in trial or not. The regulatory guidelines are modified timely. The serious injury during trial and informed consent form are recently modified. This review article put all the essential things for the reader to get enough idea about a clinical trial in India, how it is conducted, which regulatory body involved in clinical trial etc.

**KEY WORDS:** CLINICAL TRIAL, CTRI, DCGI, ETHICS COMMITTEE, ICH-GCP.

## INTRODUCTION

This review gives an overview of clinical trials and the phases involved in a clinical trial. The reader can understand the clinical trial with its importance in India. The regulation of trials in India and the various government bodies that play important roles in the trial are described in this paper. Talking about pre-clinical research gave a satisfactory statement about drug safety but it did not give any clue regarding drug interaction with the human system or its overall action on humans. Clinical research offers the study of the drug on the human body, for this, researchers develop the clinical study design with various aspects to perform. For this, there are clinical research phases and initiate the INVESTIGATIONAL NEW DRUG PROCESS. This is the step that must be done before commencing clinical research.

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The design of clinical research is made in such a way that it answers the questions related to drug products. For this, it is necessary to implement the plan and this plan is called PROTOCOL. The protocol can be made by either researchers or the manufacturer of the drug. For starting this clinical trial researchers must have basic pre-information regarding the drug and how the study be conducted to get a satisfactory result. Designing of the clinical study is nothing but, Researches have to decide basic things like duration of study, selection criteria for the subject i.e., who can involve in the study, the participants, the control group to avoid bias in future and most importantly drug administration to the subject, lastly the evaluation of data obtained. The clinical study was performed in four phases (Mahan 2014; FDA 2020).

A clinical trial is a scientifically controlled study which conducts to assess drug safety and efficacy in the human subject. This study has many pros, developing a new treatment that has a big beneficial effect over already existing therapy, the new treatment is equal to standard treatment etc (Mahan 2014). The role of the US-FDA begins after the completion of pre-clinical evaluation and safety. This

practice must be stuck to good clinical practices (GCP) (Browne et al. 2014). The criteria that must be assured during the experimental study are the well-being of the human subject, sampling, range of masking, randomization trial i.e., RCT, assessing endpoints, and evaluation of results. All of these are necessary parameters to assess the effectiveness of drugs and also provide information related to adverse drug effects (Ambroz et al. 1981; Haahr et al. 2006; Esource.com 2022).

The RCT study involves study design like the parallel, cross-over, cluster or factorial study design. Likewise, evaluation of the hypothesis involves superiority and non-inferiority (Esource.com 2022). In this study, people were randomly assigned to different treatments under the experiment. The ideal randomization increases the statistical result and reduces the bias (Arvins 1998). A clinical trial involves different phases that are- phase I, phase II, phase III and phase IV. Phase I is also known as the "earliest phase" phase II also known as the exploratory trial, phase III is known as the "later phase" and phase IV is called the post-marketing phase. There is one more phase that happens at an earlier stage of all known as phase 0. In this phase drug, ADME and pharmacodynamic studies are performed (Esource.com 2022).

In phase I there is the assessment of drug safety, in phase II effectiveness of the drug, and finally, the affirmation of both safety and effectiveness are assessed in phase III. Phase IV is the post-marketing survey that is also known as Pharmacovigilance (DeMets et al. 2015; Esource.com 2022). There is a necessity for conducting a clinical trial, to ensure the drug safety, quality and betterment of the patient's life. There is an elevation in conducting a clinical trial in India. The primary duty of the government is to ensure the well-being of the human subject that participate in the trial experiment and to maintain international standards. The clinical trial conducted in India should be as per ICH guidelines and follow schedule Y.

**Below in the table there is the overview of clinical trial phases:**

| Clinical trial phase | Description | Reference |
|---|---|---|
| Phase 0 | This phase is also known as the exploratory trial. This phase lack therapeutic and diagnostic goal. Phase 0 involves less no. of human exposure i.e., about 10 human subject.<br>Duration – 1 week<br>Human participants – 10<br>Drug dose- sub therapeutic<br>Study – pharmacokinetic & pharmacodynamic<br>This study commence before the study of dose elevation, safety criteria and tolerance.<br>Advantages: This study useful to remove drug therapy before going to phase second, it is used to evaluate new drug ADME and pharmacodynamics in humans, help to minimize the drug development expenses and time<br>Disadvantages: This phase does not have any potential therapeutic goal, lack of active involvement of human subject to participate; it may delay sometimes or exclude patients from other clinical trials that may possess potential of therapeutic goal. Un-availability of sensitive analytical method this may cause limitation to this trial, Because it necessary in micro dosing. | (Collin et al. 2007; Marchetti et al. 2007; Le Tourneau et al. 2009). |

India has the second largest pharmaceutical market in Asia that a rate of US$ 5.40 billion and its growth rate is about 9% per year. There are around 1.2 billion people in India. And about 1.5 % of clinical trials were registered at the national institute of health, the USA from India in the year 2013. There is a wide scope of clinical research in India but with this, there is a need to follow international and national rules, regulations and guidelines that are associated with clinical research. A clinical trial should be conducted by adapting ICH –GCP rules in India (World Medical Association 2013; Saxena et al. 2014). GLP i.e., Good Clinical Practice is the international ethical standard for commencing biomedical research that involves the human subject in their experimental study. This standard ensures the rights, well-being and safety of the person who is participating in research work. This is not specific but it is general and can be applied to all the protocols (Saxena et al. 2014).

| Clinical trial phase | Description | Reference |
|---|---|---|
| Phase I | This phase is used to assess the administration of drug dose with repetition criteria, it also used assess the maximum tolerated dose of the drug. The side effect of drug can also be evaluated in this phase. Most important thing is drug safety that can be evaluated in phase I. the dose of drug can be increased if it did not show any possible side effect, so that researchers can decide safest dose to therapy. Mainly healthy volunteer choose for study in experiment, in some cases the subject with disease may also required for the different kind of study.<br>Human participants- 20-100<br>Drug dose- Ascending dose<br>Drug toxicity curve and dose efficacy curve obtained by this study. This includes single and multiple ascending doses. There are two types of escalation method one is rule based and another is model based. | (Storer 1989; Jonas et al. 2007) |

| Clinical trial phase | Description | Reference |
|---|---|---|
| **Phase II** | Phases I and II are consider to be most perfect method to evaluate dose MSD that means, the maximum dose were drug is safest to administer and found to be non-toxic with desire therapeutic response. However phase I mainly target on MTD. Phase II trial help to assess the drug potential efficacy and therapeutic usefulness for the particular disease. Human participants:100-300,Study purpose- to assess how drug work and again there is evaluation of the safety of drug. Drug dose- therapeutic drug dose that find out during phase I is administered here. This study divided into two parts i.e., pilot clinical trial for efficacy and safety testing in diseased subject. Another one is rigorous trial made to demonstrate efficacy. The drug development step is terminated here if drug showing any toxic effects. This phase involve human subject showing many exclusive aspects than phase III. The randomized clinical trial design and the case studies are involved in this phase. The single stage and multi stage concepts are associated with phase II. The adaptive clinical trial is based on the accumulated data of interim that have been used for flexibility study and testing of efficacy. This is very helpful during trial to modify the design. | (Gehan 1961; Coffey et al. 2008; Brannath et al. 2010) |

**ICH in the technical requirement for human subject use in clinical research:** This platform brings various regulatory authorities together, such as Europe, Japanese, and US authorities and also the pharmaceutical company expert to share scientific and technical aspects of the registration of products (Council for International Organization Of Medical Science 2002). US FDA adopts the code of GCP initially and that is applicable to all the sponsors and investigators. Other countries don't have such a code, some county did not accept data from other countries. Europe and Japan have their own guideline that is somewhat similar to other guidelines but not exactly the same. The person who is interested to market his product in the USA must go through their guidelines and submit clinical data.

Canada, Australia, and Nordic countries formed a huge market that involves in ICH 1990, in this WHO is the facilitator and the IFPMA (International Federation of Pharmaceutical Manufacturers Associations). The ICH guidelines were completed in the year 1996 and this involves the criteria of safety, quality and efficacy with multidisciplinary. Good clinical practice involves in the E6 chapter. This helps in analysis, to guide technical issues and provides sufficient knowledge to register. So that repetition of tests or studies can be avoided. The ICH- GCP provides the responsibilities to the ethics committee, sponsors and an investigator, along with this also gives a brief idea about protocol requirements and IB i.e., investigator brochure and trial document (Saxena et al. 2014).

| Clinical trial phase | Description | Reference |
|---|---|---|
| Phase III | This phase allow experimental clinical study with detail assessment of the treatment protocol by comparing data of new treatment with standard treatment. This is the most well known scientific investigation of the newer drug treatment. This phase is also known as pre- marketing phase. This phase consumes time and it is very expensive trial.<br>Human subject- 1000- 3000<br>This phase includes randomized controlled trials, uncontrolled trial, historical control, and no randomized concurrent trial etc. This trial is classified into two steps, one is trial after efficacy study but before the NDA submission, second one is carried out after the NDA submission but before the approval. In the year 1980 the FDA has given the guidance document which states that efficacy must be showed to prolongation of life and enhances the health related quality of patients life. | (Pazdur 2008) |

| Clinical trial phase | Description | Reference |
|---|---|---|
| Phase IV | In this phase the treatment that proven to be safe, effective and good quality are made available to general public by authorization of FDA. The public, health professional people take the health benefits of marketed drug, but there are still some lacuna i.e., not all the safety and efficacy criteria has determined. There is need to continue the evaluation of safety on risk benefit ration, for this FDA requires to permit post marketing survey. This involves all the studies other than routine survey. This post marketing surveillance give actual mechanism of action of drug on given populations. In this drug related adverse effect can be observed. Long term adverse effect of the drug can be assessing in this phase, also the healthcare expenses and outcome can be determined. This phase IV result in drug can be removed from the market or may restrict to certain parameters. | (Committee on Ethical and Scientific Issues in Studying the Safety of Approval Drugs, 2012; Faden et al.2012). |

**Ethics committee:** This committee has a role in developing a constitution and standard operating procedures that must involve the members and the appointment conditions. The ethics committee look after all the protocols of the research study, the informed consent form of the patient and other necessary documents that are associated with the research proposal. After a detail reviewing of all document's ethics committee supposed to give approval to the study and supervise the whole study (Desai et al. 2013). It also provides a safeguard to the subject who is involved in the study, the well-being of the subject is the priority of the committee. The committee must have to overlook the experimental study on daily basis. The committee must have to maintain confidentiality and also maintain all the records and documental proof. All records should be maintained until 5 years of duration after completion of the study. This

committee also has a role in examining SAE reports and sending them to DCGI. This committee allows DCGI to inspect and follow all national and international protocols (Bonthagarala et al. 2017).

**Sponsors:** A sponsor is the one who takes the responsibility for starting, managing and giving financial support to a clinical trial. The Investigator can also play the role of a sponsor performing all the duties related to the trial called the role of sponsor. Sponsors have the right to give clinical trial-related activities to any scientific body or contract research organization. Such transfer should be documented well in writing format. The sponsor should evaluate the investigator before starting the actual study, the sponsor must be a trained person, and he is having experience and knowledge in conducting the trial. The sponsor has the responsibility to assess the recruiting potential of the investigator based on previous reports, space, time, equipment, teamwork, lab facility etc. the investigator brochure must be provided to the investigator before commencing to study. Another responsibility of the sponsor is to obtain approval from DCGI and Institutional Ethical committee. The serious adverse event that happens during the trial should be recorded within the given period. The sponsor has to submit the protocol report, case report form and ICF. They have to monitor, assist, and evaluate all the data and financing (Council for International Organization Of Medical Science 2002; Bonthagarala et al. 2017).

**Figure 1: Diagrammatic representation of various laws, regulations and guidelines that plan and monitor the clinical trial conducted in India.**

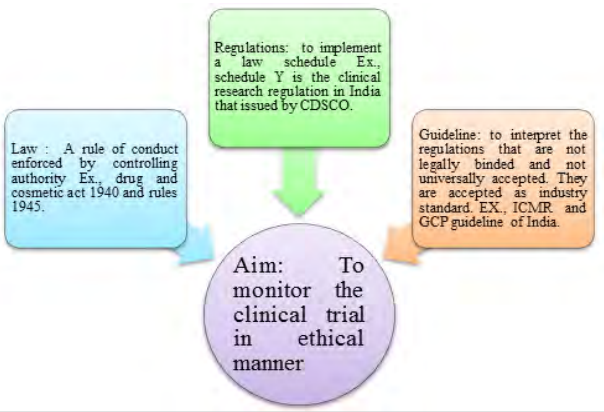


**Figure 2: The figure showing criteria that should be meet before conducting actual clinical trial in India.**


**Investigator:** The actual in charge of all the studies conducted and supervision of this whole procedure is done by the investigator. The investigator should possess the criteria to fulfil his duty is qualification first, then training experience and treatment facility related to the protocol of the study. Before commencing to trial, the sponsor and investigator must have signed an agreement on paper, on protocol, monitoring responsibilities and trial-related duties etc. The investigator has the job to obtain approval from the institutional ethics committee, to get ICF from each human subject involved in the study. Any serious adverse event that occurs during the trial, needs to be reported as early as possible in the fixed period.

Investigator does not take more than three trials at the time (Council for International Organization Of Medical Science 2002; Saxena et al. 2014). What is CTRI? (The Clinical Trials Registry-India) and need of it: The CTRI were launched on 20/7/2007, this is the absolutely free, searchable online site where clinical trial registration can be done. On this platform, all types of clinical trial-related studies can be registered. The PG theses can also be registered here. Recently there are many types of registration that can be done on CTRI Like- intervention trials, observational data, bioavailability, and also the phase IV studies. All these trials are easy to search and handle by common people from its official home page (Adhikari et al. 2018).

Medical science has developed so fast because of this there is an increase in new therapeutic measures and ultimately the method for designing the newer agents. The trials that are not ethically conducted further lead to withdrawal from the market in future therefore it is essential to create transparency and easy watch out on clinical trials for research persons and the public (Adhikari et al. 2018). In the year 2008, the editors of the biomedical journal support clinical trial registration. They had published a joint statement that states “don’t accept the unregistered clinical trial for publication in any journal from the year 2010” (Bavdekar et al. 2008; Adhikari et al. 2018). The World Health Organization (WHO) reorganize the CTRI as the primary registry in the year 2008, from this year every month data gets transferred from CTRI to the international clinical trials registry site. This is the medium where globally all clinical trial gets registered. In the year 2009, the CDSCO make it compulsory for every clinical trial that supposes to be conducted, to do their registration in CTRI first (Adhikari et al. 2018).

To maintain a smooth flow in trial registration the CTRI software was upgraded and revised all the data on dated 15th march 2011. This new software makes it possible to help the overall software paperless and there is also a facility to upload all regulatory approvals on an online basis. Furthermore, the CTRI arrange a number of workshops that involve ethics committee members, to grow the trial registration and to make awareness among people. Various ethics committees made it mandatory for trial registration. The improvement was observed when AYUSH also come to accept the trial registry. The E- Tutorial was launched in 2015. This tutorial is made to guide the process of trial registration (Adhikari et al. 2018).

The importance of CTRI in the registration of PG theses is- CTRI registration is important in the registration of

PG theses that would be helpful in raising the standard of research so that there will be a limit to the repetition of already done research work. This can act as a ready guide that answers the research questions like "how one can start research work, how one can start clinical research, student can receive information regarding protocols and strategies of research work that would be beneficial in their future work (Adhikari et al. 2018).

**The future expectation of CTRI:** The registration of the trial is not enough to fulfil all criteria, there should be the disclosure of the result is also mandatory to maintain transparency and accessibility. Because of this reason, ICMR joins the WHO on 18th may 2017 to make sure that there is a disclosure of results within one year after completion of the trial. Taking this into consideration the CTRI develop the structural framework that should involve the patient number, characterization of baseline, primary and secondary outcome, and adverse event of the drug. This protocol makes it easy to reach the public hand. Since 1st April 2018, CTRI moved towards prospective trial registration that is only for clinical studies. This is applicable to all types of clinical studies that are submitted for registration (Adhikari et al. 2018).

## CONCLUSION

The overall conclusion of the present article found that it is essential to describe the clinical research, how it was conducted and what clinical trial phases are. To understand the drug therapy that is required to pass through multiple phases before coming to market and the importance of safety and health of a population. The various regulatory bodies that ensure all experimental studies should be conducted ethically. It is essential to maintain the highest quality and standard in drug safety and efficacy evaluation parameters as it directly links with wellbeing of the population.

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# Efficacy of Kodo Millets *Paspalum scrobiculatum*: A Systematic Review

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## ABSTRACT
In recent times, when the COVID pandemic has hit the world badly, there has been a marked shortage of food, water, and other essentialities, an increase in food pricing which together with other socioeconomic impacts have eventually posed threats to agriculture, food supplies, and nutritional security all over the world. Researchers worldwide are looking for potential solutions to combat poverty and hunger issues. This review focuses on the various medicinal properties of Kodo, its uses in various fields, and prospects where it could be utilized thereafter. The findings of the present rerview revealed that the Kodo millets, *Paspalum scrobiculatum*: are nutritionally dense when compared to the number of grains consumed. They have a greater mineral content when compared to rice and wheat. Some of them weigh fifty times as much as rice. Finger millet contains thirty times the calcium content of rice, whereas every other millet has at least half the calcium content.

**KEY WORDS:** KODO MILLET, PASPALUM SCROBICULATUM, CARDIOVASCULAR DISEASES, OBESITY, FOOD.

## INTRODUCTION

Drought-resistant plants such as Kodo millet are rare, they are made up of coarse grain. Rice grass, cow grass, and millet are some of the alternate names for Kodo millet. *Paspalum scrobiculate* is a tropical African plant that was first cultivated 3000 years ago in India. The grain is encased in a testa that must be removed before preparation (Krishnan et al. 2012; Baghele et al. 2021).

Grain has 98.3 percent protein, 1.4 percent fat, 65.6 percent carbs, and 2% ash. Fiber The overall fiber level of the grain is fairly high throughout. Kodo-millet has a lower Phosphorus (P) concentration than other millets, and it has a significantly higher antioxidant capacity than virtually other millets and common cereals (Ratnavathi 2017). The grain is recommended as a rice supplement for those with diabetes (Bhat 2018). India's Uttar Pradesh, Rajasthan, Bengal West, Tamil Nadu, Andhra Pradesh, and Madhya Pradesh grow this crop (Bhat 2018; Baghele et al. 2021). Throughout the year, Kodo millet is produced on 1.97 lakh hectares over a vast region, with a total gross output of about 0.84 lakh tonnes and a yield of 429 kg per hectare (Pradesh 2011). Kodo millet is mostly grown in hot and temperate regions. That is drought resistant and, as a result, may be grown in areas where rainfall is scarce and erratic. It's wonderful to thrive in areas where yearly precipitation is just 40 to 50 cm (Tadele 2016). Because of the nutritional and physiological benefits they provide to customers, v. millets have become a popular food raw material alternative for major cereals in recent years (Baghele et al. 2021).

The coarse grains Kodo millet (*Paspalum scrobiculatum* L.) and small millet (*Panicum miliare* L.) are mostly grown in India, China, the Soviet Union, Japan, and Africa (Kumar et al. 2016). In 1998, coarse grain output totaled 902 million tonnes, accounting for roughly 44% of total world cereal production. India produces 3 I million tonnes of coarse grains, ranking third in the world behind the United States and China, with a share of 3.4 percent. In 1950-5, the area under coarse cereals was 37.6 million hectares, rising to a peak of 47.34 million hectares in 967-68 before steadily declining to 30.00 million hectares in 2000-0, of which just 2% is irrigated (Kulkarni et al. 2006; Kumar et al. 2016; Baghele et al. 2021). In Madhya Pradesh, India's tribal area, Kodo is a staple meal (Sharma and Mandhyan 1992). The bran layer on the surface of the Kodo kernel makes it tough to digest. The final product is more pleasant and digestible if Kodo is dehusked and then polished to remove the bran layer. Millets are also acceptable for diabetic diets, although their distinctive flavor and difficulties in the processing are limits to their use in diets (Baghele et al. 2021).

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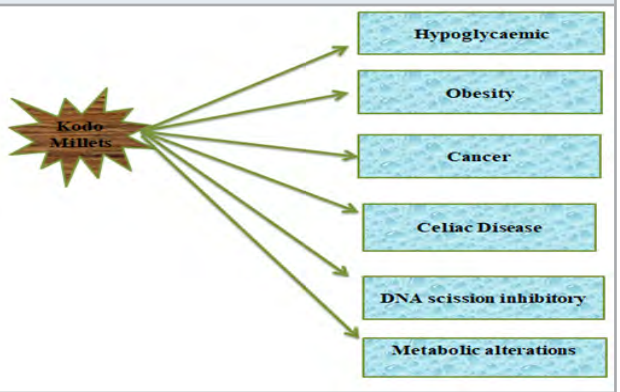
Kodo millet is nutritionally equal to other common grains, and in certain ways, such as minerals and fiber, it is even superior to rice and wheat (Gopalan et al. 2018). Hand/foot pounding is still used in the milling of Kodo millet. The requirement of the day, however, is to make use of the possibilities of identifying processing methods and suitable machinery for Kodo millet processing (Baghele et al. 2021). Physical and technical characteristics of biological materials are critical not only for classifying them into subgroups but also for developing handling and processing equipment and procedures. Cleaning, grading, categorization, shipping, aeration, drying, and storage are all part of post-harvest processing for value addition. Knowledge of grain characteristics and qualities is required for successful post-harvest equipment design (Liu et al. 2017; Baghele et al. 2021).

**Bioactive substances in Kodo millets:** Some grain components have bioactivity in relation to the nutrients they offer. Phytochemical compounds abound in Kodo millet grains. Tannins, phenolics, flavonoids, alkyl resorcinol, and coumarins are phytochemicals. Phenols are responsible for the oxidative quality of plant-based foods' flavor, texture, color, and taste (Naczk and Shahidi 2004). They are usually found in Bran and have nutraceutical benefits. Millet is more than simply an interesting alternative for the more popular grains. The grain also contains phytochemicals such as phytic acid, which is considered to lower cholesterol, and phylate, which is linked to lower cancer risk (Coulibaly et al. 2011). Certain health advantages have been ascribed in part to the wide spectrum of potential medicinal substances known as phytochemicals, which may be found in foods such as millets in high concentrations of antioxidants (Izadi et al. 2015; Baghele et al. 2021).

**Table 1. List of bioactive substances responsible for curing disease.**

| Bioactive substances | Disease cure |
|---|---|
| Saponins | Improve immune function by stimulating of production of T cell (Marciani et al. 2000) |
| Flavonoids | Cardiovascular, (Sharma et al. 2018) |
| Glycosides | Diuretic effect, (Sharma et al. 2017) |
| Alkaloids | Antioxidants, (Singh et al. 2016) |
| Triterpenoids | Diabetic complications, (Nazaruk and Kluczyk 2015) |
| Steroids | Reduce systemic inflammation, (Hill et al. 2020) |
| Tannin | Tonsillitis, Pharyngitis, (Kamal 2014) |
| Phenolic acids | Cancer treatment, (Abotaleb et al. 2020) |

**Figure 1: Role of Kodo Millets in treatment.**




**Hypoglycemic properties of Kodo millet:** In a research published in 2013, Neelam et al. (2013) found that when Sewai upma and Idli were mixed with Kodo millet, the mean quantity of glucose and GI were reduced (60%). In the control Idli and Sewai upma, the CV percent of the inter-individual variation was determined to be 4.03 and 4.98, respectively. Similarly, the GI values in the Kodo-dependent items were low (Kodo Idli 3.98 and Kodo upma 3.53). Lesser CV percent values of average blood glucose levels indicate variations across participants, which offers useful statistics for comparing the accuracy of various variables (Farugui et al. 2013; Liu et al. 2021).

The lower CV percent number indicates that the research participants were a homogenous group. Cereals and millets are the most abundant sources of carbohydrates and a key source of human energy. Carbohydrate foods, particularly rice and wheat (60-65 percent), supply the majority of energy in the Asian Indian diet (Amadou et al. 2013). It's critical to comprehend the glycemic reaction of such common meals, especially for insulin-resistant individuals (Mohan et al. 1986; Zhang and Hamaker 2009; Farugui et al. 2013; Liu et al. 2021). According to Brand-Miller et al. (2003), the glycemic index value may be divided into three categories: a hundred (high GI food). According to studies, white rice and extruded wheat flour products have high GI values, therefore traditional Idli (control), which is generally made of rice, and Sewai upma, which is made of refined wheat flour, may be classed as high GI foods and are not advised for diabetes patients. In research conducted by Neelam et al. (2013), control Idli and Sewai upma were replaced with Kodo millet (60%) and the GI was reduced by 15% and 9%, respectively (Liu et al. 2021).

This would be especially important in Southeast Asia, where diabetes is one of the most common health issues

and where the diet is often heavy in carbohydrate cereals (30). The crude fiber content of Kodo millet (8.5 g/100 g) was found to be higher than that of white rice (0.2 mg/100 g) and refined wheat flour (0.3 mg/100 g), which might explain why Kodo-based products have a lower GI than controls when carbohydrate content is changed (Liu et al. 2021). The presence of dietary fiber, type of carbohydrate, nature of starch granules, the physical shape of food, and processing, according to Ludwig et al. (1999), impact the glycemic effect of food. Furthermore, the ratio of amylose to amylopectin in starch, as well as an alpha-amylase inhibitor, decreases the GI of meals by delaying starch digestion (Liu et al. 2021).

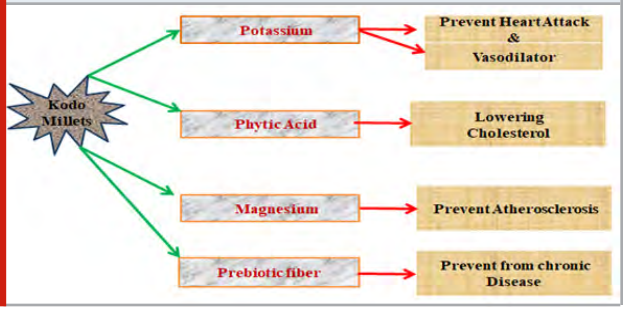
Incorporating high-fiber foods into a variety of goods can help to decrease a product's glycemic reaction. Dietary fiber lowers the postprandial blood glucose response simply by slowing carbohydrate absorption in the small intestine due to the development of a viscous gel. Millets outperform other cereals in terms of nutrients. Millet is high in dietary unavailable carbohydrates, beta-glucan, and soluble sugars, all of which help with glucose metabolism (Augustin et al. 2002; Liu et al. 2021). Kodo millet's therapeutic impact on reducing postprandial blood glucose response is most likely owing to its highly viscous soluble fiber, which is not hydrolyzed by digestive enzymes. Soluble fiber causes extremely viscous intestinal contents with gelling characteristics, which may delay absorption in the intestine. The quantity of carbohydrates ingested, as well as the GI of the carbohydrate diet, influences glucose and insulin response. Glycemic load represents both of these factors. The combination of low cereal dietary fiber consumption and high GL was linked to a higher risk of diabetes and coronary heart disease in women (Vijayalakshmi and Radha 2006; Liu et al. 2021).

**Obesity:** Obesity is India's fastest-growing problem, and it's linked to a variety of chronic diseases, including diabetes and cardiovascular disease. According to new research, eating a high-fiber diet lowers the risk of obesity (Alfieri et al. 1995; Liu et al. 2021). Dietary fiber-rich foods enhance intestinal function and delay the digestion and absorption process, lowering the risk of chronic illnesses. Millets have a greater dietary fiber content than other cereals, with 22% compared to 12.6 percent for wheat, 4.6 percent for rice, and 13.4 percent for maize. According to Chethan et al. (2007), finger millet grain contains 15.7 percent insoluble dietary fiber and 1.4 percent soluble dietary fiber. Finger millet comprises 22.0 percent total dietary fiber, 19.7% insoluble dietary fiber, and 2.5 percent soluble dietary fiber (Shobana and Malleshi 2017). Dietary fibers are divided into soluble and insoluble fibers, as we all know (Arya and Bisht 2022).

According to studies, eating a high-fiber diet improves bowel function and lowers the incidence of obesity by increasing digestion and absorption in the body, lowering the risk of chronic illnesses. Millets aid in weight control and the reduction of obesity by satiating appetite. Millets' high fiber content aids in the relief of constipation, gas, bloating, and stomach cramps. The retention of gastrointestinal diseases such as ulcers and colon cancers can be reduced with proper digestion and absorption (Reddy 2017; Arya and Bisht 2022).

**CVD (Cardio Vascular Disease):** Kodo Millets demonstrated that eating porso-millet protein concentrate influenced plasma lipid levels, with plasma high-density lipoprotein cholesterol and adiponectin levels notably raised (Kyung-Hye et al. 2015). Kodo millets are also high in magnesium, which has been linked to a lower risk of a heart attack. Millets are high in phytochemicals, including phytic acid, which helps reduce cholesterol and prevent cardiovascular disease by decreasing plasma triglycerides (Lee et al. 2010). According to studies, eating whole millet grains daily lowers the risk of cardiovascular disease. Kodo Millets are one of the greatest grains to include in your diet if you want to protect your heart, which is a concern that everyone has. Magnesium, which is abundant in Kodo Millet, is a vital element for lowering blood pressure and reducing the risk of heart attacks and strokes, especially in the case of atherosclerosis. Millets are also high in potassium, which acts as a vasodilator to lower blood pressure even further. They produce enterolactone, a substance that has been shown to protect against heart disease and several types of breast cancer when fermented (Reddy 2017; Arya and Bisht 2022).

**Figure 2: Role of Kodo millets in CVD**




**Cancer:** The antinutrients phenolic acids, phytates, and tannins found in Kodo Millets have been shown to help reduce the risk of colon and breast cancer. The phenolics in Kodo millet are effective in preventing cancer development and progression *in vitro*. Disrupting agents, in particular, prevent cancer from starting by preventing cellular target molecules, such as DNA, from interacting. In a study, phenolic extracts from Kodo millet inhibited the growth of human colon adenocarcinoma cells HT-29 in a time and dose-dependent manner (Chandrasekara and Shahidi 2011). Kodo millets contain linoleic acid, which has anti-tumor properties. Sorghum is widely known for its anti-carcinogenic properties. Polyphenols and tannins present in sorghum have anti-mutagenic and anti-carcinogenic characteristics and can work against human melanoma cells as well as advantageous melanogenic activity (Grimmer et al. 1992; Arya and Bisht 2022).

Sorghum intake was associated with a lower incidence of oesophageal cancer in China and other areas of the world (Rensburg 1981). The scientists investigated 21 villages in each nation over six years and discovered that sorghum intake was associated with reduced oesophageal cancer

mortality than wheat and maize consumption. Many of the antioxidants contained in millets may clear up other toxins in your body, such as those in your kidney and liver, in addition to their positive effect on neutralizing free radicals, which can cause cancer. By encouraging appropriate excretion and neutralizing enzymatic activity in those organs, quercetin, curcumin, ellagic acid, and other helpful catechins can assist to clear your system of any external agents and poisons (Reddy 2017; Arya and Bisht 2022).

**Celiac Disease:** Celiac disease is a genetically predisposed illness brought on by gluten consumption. Since Kodo millets are used to help reduce celiac disease by reducing pain caused by gluten-containing cereal grains, they are gluten-free (Saleh et al. 2013). Regulating the digestive system can help with nutrition retention and reduce the chance of more serious gastrointestinal issues like gastric ulcers or colon cancer. Fiber content in Kodo millets aids in the elimination of diseases such as constipation, excessive steam, bloating, and cramps (Arya and Bisht 2022).

Celiac disease is an immune-mediated enteropathic illness that is often caused by gluten consumption in susceptible individuals (Catassi and Fasano 2008). In the grain food community, a gluten-free diet has a significant impact on food consumption. It can help gluten-free dieters replace gluten-containing grains like wheat, barley, and rye with gluten-free grains including rice, maize, sorghum, millet, amaranth, buckwheat, quinoa, and wild rice (Thompson 2009). Because Kodo millets are gluten-free, they have a lot of food and beverage potential. They'll help fulfill the growing demand for gluten-free meals and would be perfect for celiac disease sufferers (Arya and Bisht 2022).

**Wound healing:** Under ether anesthesia, a 4 cm2 (2 x 2 cm) excision incision on the shaved back of rats were made to test the effect of Kodo millet on rat dermal wound healing. For 16 days, finger millet or Kodo millet flour (300 mg) was administered topically once a day as an aqueous paste. Some biochemical parameters such as protein, DNA, collagen, and lipid peroxides were estimated using the granulation tissue produced on days 4, 8, and 12. Protein and collagen content increased significantly, whereas lipid peroxides decreased significantly. Biophysical factors such as contraction rate and epithelialization time were also investigated. In Kodo millet and finger millet-treated rats, the rate of contraction was 88-90 percent, compared to 75 percent in untreated rats. In comparison to untreated (16 days) rats, finger millet (13 days) and Kodo millet (14 days) treated rats required fewer days to complete wound closure. The findings suggest that finger millet and Kodo millet may have a therapeutic effect in speeding wound healing (Hedge et al. 2005; Arya and Bisht 2022).

DNA scission inhibitory activity: The initial stage in many biological situations, such as cancer and aging, is the irreversible change of DNA as a result of oxidative stress. Base changes, the formation of base-free sites, DNA-protein cross-linkages, strand breakage, and aberrant chromosomal architecture are all examples of free radical-mediated DNA damage (Valko et al. 2004; Arya and Bisht 2022).

The hydroxyl radical may extract hydrogen from the deoxyribose sugar moiety as well as pyrimidine and purine DNA bases, resulting in single strands. Breaks in the double strand that occur on both strands near each other might be caused by hydroxyl radical assaults, which could result in cell death. Two dosages of raw phenolic derivatives (0.25 and 0.5 mg/mL) were tested for free radical-mediated DNA strand scission prevention and the percentage of supercoiled DNA retention was measured in a study done by Chandrasekara and Shahidi (2010). Peroxyl and hydroxyl radicals both damage DNA strands, according to the research. Antioxidants and many other methods have proven polyphenols to be chemopreventive (Fresco et al. 2006; Zgórka et al. 2022). Millet grain phenolic extracts, in particular, have proved to be effective. DNA scission was produced by the inhibitory effect against peroxyl radical at both doses examined. Kodo millet inhibited DNA breaking completely and was comparable to 0.5 mg/mL ferulic acid in this regard. It's worth noting that Kodo millet's inhibitory behavior against peroxyl radical-mediated DNA split was the same (97 percent ) (Fresco et al. 2006; Zgórka et al. 2022).

Madhujith and Shahidi (2007) earlier reported that phenolic extract of barley grains suppressed DNA break caused by peroxyl radicals. At a concentration of 4 mg/mL, supercoiled DNA scission-induced peroxyl radical inhibition of barley extract ranged from 78 to 92 percent. Supercoiled DNA retention toward hydroxyl radical driven oxidation ranged from 30% to 90% in the presence of Kodo millet grain extracts. It has been found that a similar quantity of Kodo millet extract (0.5 mg/mL) suppressed DNA breaking induced peroxyl radicals 2-3 times more than hydroxyl radicals. Madhujith and Shahidi (2007) found that phenolic extracts of several barley varieties were considerably more effective against DNA scission inhibition induced by peroxyl radicals than hydroxyl radicals. Parallel to the reaction with purine and pyrimidine bases, the deoxyribose backbone of the DNA molecule can be treated with a hydroxyl radical, resulting in few commodities with oxidative (Arya and Bisht 2022).

To prevent supercoiled DNA breaking induced by hydroxyl radicals and therefore inhibit the formation of hydroxyl radicals, two ways of chelating Fe ions are required to begin and catalyze the breakdown of $H_2O_2$ or the scavenging of $H_2O_2$ itself are required. The capacity of phenolic compounds to scavenge hydroxyl radicals produced in the system is the second mechanism. Two types of Kodo millet were effective metal chelators. In previous research, we discovered that phenolic extracts from Kodo millet effectively scavenge $H_2O_2$ and hydroxyl radicals *in vitro* systems (Chandrasekara and Shahidi 2011; Arya and Bisht 2022).

**High fat diet-induced metabolic alterations:** The underlying adiposity of chronic low-grade inflammation is one of the most important interaction services that contribute to comorbidities such as insulin resistance, diabetes type II, and hepatosteatosis (Asrih and Jornayvaz 2013). The recent alarming increase in the incidence of obesity highlights the need for strategies, resources, prevention, and preventative

measures to manage this burden. In this regard, the search for new functional foods and dietary patterns that may be able to cure or prevent obesity has exploded (Baboota et al. 2013; Arya and Bisht 2022).

Increased intake of whole-grain cereals is a significant improvement in daily eating habits and has been shown to improve the body's metabolic profile. The polyphenol and dietary fiber content of these cereals are largely focused on, but not limited to, their health advantages (Baboota et al. 2013). Millets appear to have higher nutritional content than big grains in terms of phenolic acids, dietary fibers, and antioxidant capabilities (Kumar et al. 2018). KM is more effective at quenching free radicals than wheat and rice, the world's most popular cereals. When compared to rice products, KM food items have lower TG and C-reactive protein levels. kM has also been shown to provide a variety of health advantages, including glucose-lowering, wound-healing, and anti-obesity effects. However, research work is done on the role of millet polyphenols, particularly KM, in preventing HFD-induced obesity and other abnormalities. Kodo millet is well-known for its numerous health advantages. A polyphenol-rich extract from them has been shown to have antioxidant and hypoglycemic properties in several investigations (Arya and Bisht 2022).

Nonetheless, the protective role of a polyphenol-rich extract from Kodo millets in combating obesity caused by a high-fat diet has yet to be studied. Khare et al. (2020), investigated the role of polyphenol-rich extracts in decreasing lipopolysachharides-induced inflammation in murine macrophage cells and attenuating high-fat diet-induced metabolic complications in Swiss albino male mice in research. The results showed that polyphenol-rich extracts from Kodo millets had a greater polyphenol content, which prevented obesity. Furthermore, the polyphenol-rich extracts from Kodo millets were more effective in preventing weight gain, hepatic steatosis, diabetes, hypertrophy of adipose tissue & systemic inflammation (Khare et al. 2020).

According to the findings, polyphenol-rich extracts from Kodo millets might be utilized to make functional diets or nutraceuticals to combat obesity and comorbidities (Khare et al. 2020). The microbiota of the gut has a key role in obesity and the inflammatory diseases that accompany it. In research done by Khare et al. (2020), the caecal material showed a greater percentage of Bacteroidetes to Firmicutes with KM-PRE, which is believed to be an indication of weight reduction. Furthermore, KM-PRE fed mice had low levels of LPS in their circulatory system, but HFD fed mice exhibited higher levels (Magne et al. 2020).

LPS is produced in the gastrointestinal tract by Gram-negative bacteria, particularly pathogenic types linked to dysbiosis in the gut microbiota, and is transported to the bloodstream in obese animals and humans owing to weakened gut barrier function (Chakaroun et al. 2020). This systemic LPS has been proven to promote low-grade inflammation in obesity and related comorbidities. 40 KM-PRE protected against the degradation of gut barrier control caused by HFD feeding by avoiding these negative alterations. Nutritional phenols are found as glycoside conjugates that are converted into physiologically active aglycones by gut bacteria and free sugar could have been used to encourage SCFAs by gut bacteria (Duda-Chodak et al. 2015).

In one research, KM-PRE, a marker of increased gut bacterial dysbiosis, restored the decrease in propionic acid produced by the HFD. Some polyphenols or polyphenol-rich extracts have been shown to activate Bifidobacteria and Lactobacilli, while others have been shown to stimulate *Akkermansia muciniphila*, the primary bacterial actors in HFD-induced obesity (Ley et al. 2006). The expression of PPAR and SREBP-1c in the vWAT of mice fed with KM-PRE was shown to be lower. This is attributed to the Mitigation of adipocyte hypertrophy and hyperplasia, as apparent from histology, which induces greater PPARγ expression, C/EBP-alpha and SREBP-1c the indicators of diet mediated obesity (Moseti et al. 2016).

Polyphenols like coumaric acid, quercetin, catechin, chlorogenic acid, resveratrol, and curcumin have been shown to reduce PPAR synthesis and efficiently respond to insulin, according to Khare et al. 2020. 44-46 SREBP-1c expression has been linked to an increase in hepatic lipid buildup. In a biochemical study conducted by KM-PRE, reversing the HFD resulted in a rise in liver SREBP-1c expression as well as a decrease in liver lipid, indicating that it has a favorable influence on hepatic steatosis as well as enhanced insulin sensitivity. The KM-PRE-PR treatment reversed HFD-induced glucokinase and G6Pase levels in the liver, as well as the HOMA-IR and QUICKI indices further, confirms this finding (Magne et al. 2020).

## CONCLUSION

The findings of the present study revealed that the Kodo Millets are nutritionally dense when compared to the number of grains consumed. They have a greater mineral content when compared to rice and wheat. Some of them weigh fifty times as much as rice. Finger millet contains thirty times the calcium content of rice, whereas every other millet has at least half the calcium content. Rice isn't even close to the competition when it comes to iron content, thanks to the abundance of foxtail and small millet. Even the most fortunate rice, ironically, lacks this critical vitamin.

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**Conflict of Interests:** Authors declare no conflicts of interests to disclose.

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# Fish Poly Culture in Domestic Wastewater Ponds: A Step Towards Protein Rrecovery and Pollution Reduction

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## ABSTRACT

Reclamation or recycling wastewater is an alternative to the gradual degradation of natural water resources. Reused or domestic sewage is highly loaded with nutrients, suspended solids, organic and inorganic matter, and microorganisms that provide natural food for several species of edible fishes. The effluent contains excessive nutrients which may increase the growth of aquatic plants and stimulate the production of natural food for fish. Oxidation ponds or stabilization ponds in the tropics are recognized as effective and economical units for the treatment of domestic sewage as well as biodegradable industrial wastes if managed properly. The driving force in a waste oxidation pond is solar energy utilized by active continuous photosynthesis. The action of sunlight on algae in the pond enables them to grow and rapidly consume the nutrients contained in the sewage. The algae and bacteria play an inter-dependent symbiotic role in these ponds, while the algae use the nutrients and carbon dioxide by bacterial decomposition, the bacteria make use of the oxygen liberated by the algae during photosynthesis, consequently increasing the rich natural biomass for the fishes. Updated compiled information in this review article suggests that domestic waste-water aquaculture is one of the best alternative ways to remove eutrophication as well as increase the culture of poly carps. This domestic sewage-purification cum reclamation bioprocess can be one of the cheapest methods, where natural sunlight, tropical conditions and biological parameters if managed judiciously, can be recycled and reclaimed for economically viable fish culture.

**KEY WORDS:** AQUACULTURE, DOMESTIC SEWAGE, EUTROPHICATION, RECLAMATION.

## INTRODUCTION

World–wide attention has been focused on recent events in many parts of the world and on the problem of shortages and mal – distribution of food resources. The increasing population explosion coupled with apparent climatic changes and the rapid skyrocketing of oil prices has contributed to the worsening of the food situation to the conclusion that the problem will almost certainly become more critical in the future. To meet the critical shortage of world food supplies, intensive research is underway in several countries to develop technology for the massive and economic production of protein from the natural resources which at present are adequate, though dwindling fast.

Parallel problems receiving attention are those of preserving the quality of the environment and the proper management of limited resources, while fisheries constitute a small part of the gross national product (GNP) in most nations in Asia, their role in national development is of considerable significance in terms of job employment, foreign exchange earnings, food supply and more importantly socio-economic stability of the rural area where the majority of Asian populations live.

Reviews of the current status of the fisheries industries show that Asia still remains a center of fishery and aquaculture activities contributing more than 50% of fish production. Fishing and aquaculture play a significant role in contributing fish protein to a large population many of whom suffer from chronic malnutrition providing direct employment to fishermen and indirect employment in fisher stability–related industries; assisting in the socioeconomic stability of the rural area and in recent years in assisting developing countries earn foreign exchange through increasing export of high – priced commodities, (Ali et al 2021).

Unlike cereal protein, fish contains essential amino acids such as lysine and thus serves as an efficient supplement

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to the low-protein, high-carbohydrate diet of developing nations in Asia. Fish contribute a relatively large share of the animal protein intake and account for 33% of the meager animal protein consumed by the average Asian people. Moreover, the low official fish consumption rate presented by the United Nations Food and Agriculture Organisation (14.7 kg per capita, 1999-2006), does not reflect a reality where significant catches are unreported and therefore under-estimated in official statistics. Fish and other types of seafood are an important source of protein worldwide. Globally, they comprise about 6 percent of dietary protein, but for billions of people, fish account for up to 20 percent of the average per-capita intake of animal protein (FAO, 2014).

Water, which is one of the most vital resources for all kinds of life is also the resource which is most adversely affected both qualitatively and quantitatively by all kinds of human activities on land, air and water. It is known that the aquatic ecosystems are most delicately balanced and get easily disrupted by various human activities. The micro and macro communities in nature are orderly and play an important role in keeping the water healthy and acceptable for various uses. However, pollution of any kind, affects these communities, hindering their effective utilization. In various types of human activities, sewage disposal continues to be the most ominous one, especially in developing countries, (Vollenweider, 1968; Mara, 1976' Shuval, 1977; Biswas and Arar, 1988; Ali 1988 Ali, 1991, Ali 2000, Ali et al., 2020, 2021, Shobana et al., 2021).

With increasing urbanization and rapid growth in population, the reuse of wastewater in agriculture and aquaculture plays an essential role in reducing waste products and saving water especially when there is fast depletion of freshwater resources. Oxidation ponds or stabilization ponds are recognized as effective and economical units for the treatment of domestic sewage as well as biodegradable industrial wastes. The driving force in a waste oxidation pond is solar energy utilized by active continuous photosynthesis. The action of sunlight on algae in the pond enables them to grow and rapidly consume the nutrients contained in the sewage.

The algae and bacteria play an inter-dependent symbiotic role in the oxidation ponds, while the algae use the nutrients and carbon dioxide by bacterial decomposition, the bacteria make use of the oxygen liberated by the algae during photosynthesis, consequently increasing the rich biomass. This mechanism of sewage purification is one of the cheapest methods, where natural sunlight, tropical conditions and biological oxidation are used. Due to these biological processes taking place in special ponds, the chances of recycling and recovery of nutrients from sewage are very high, (Chakrabarti et al, 2011; Lahiri et al. 2018; Bojarski et al., 2020, Ali et al., 2021).

Sewage may be defined as "a cloudy or dark fluid with a very foul smell, arising out of domestic wastes containing mineral and organic matter either in solution or heavy particles of solid matter floating or in suspension or in colloidal and pseudo-colloidal forms in the dispersal of state. Sewage may vary considerably in composition and strength from place to place owing to marked differences in dietary habits and consumption. The strength of sewage is determined by the amount of oxygen to oxidize the whole organic matter content present in it (Modak, 1938; Mara, 1976).

Domestic treated sewage in the tropics is a rich source of nutrients, hence has been used for intensive aquaculture as well as abating the eutrophication of waters, but is often neglected in India due to well-known reasons. Disposal of sewage is a worldwide problem and consequently has received great scientific attention. Raw domestic sewage contains a huge amount of organic and inorganic compounds along with nutrients (WHO, 1989; Kaur et al., 2018; Hoffmann et al., 2020), which can be recycled. Hence, the enormous nutrients in sewage water can be used to culture fishes (WHO 1989, Ghosh, 2018 Ali 2000). Fish farming using domestic sewage water has been practised for decades by many cultures. In sewage water, an enormous amount of nutrients serves as an ideal fertiliser for phytoplankton and zooplanktons to increase and flourish the productivity of the aquatic ecosystem, which eventually serve as valuable food for the fishes, (Mandal et al, 2015; Mandal et al, 2018; Bunting and Edward, 2018; Prakash & Verma 2020; Shobana et al., 2021).

Fish farming using domestic sewage water has been experienced for hundreds of years by many countries across the world, (WHO, 1989, Nandeesha et al., 2002; Jana et al., 2018). It is one of the best alternative ways to treat domestic waste for the fish culture. It also involves one of the cheapest and eco-friendly processes to remove excessive nutrients like phosphorous and nitrogen to maintain a balanced food cycle of the ecosystem, (An et al., 2003; Wang et al., 2013; Manea, & Ardelean, 2016; Yang et al., 2019, Ali 2000, Ali et al., 2020; Li et al., 2021).

In India, fish culture practices in freshwater ponds utilised with domestic sewage are of rather a recent origin. Several successful attempts were made by various investigators on the productivity of sewage ponds in several southern and eastern states of India. The use of sewage effluent or treated sewage for raising fish from ponds in India was advocated by Hora (1944), Pillai et al. (1945), Ganapati and Chacko (1951), Bhatia et al. (1970). Apart from the fish culture in sewage-fed ponds made some observations on the hydrological conditions of newly constructed sewage-polluted ponds in Madurai, Saha et al. (1970) have described the chemical nature of raw sewage and hydrological conditions in sewage–fed ponds.

In view of the present rate of generation of sewage and already loaded aquatic resources, it has been increasingly recognised that disposal of sewage into fresh waters possess a variety of hazards. In developed countries, the sewage is handled by well-developed organizations, treated at various levels and ultimately disposed to freshwater or recycled. The reuse of municipal and industrial sewage has become an attractive option to avoid the effects caused by sewage on the freshwater ecosystem. On the other hand, in developing countries, sewage disposal still remains a major problem,

despite the fact that low-cost technologies for disposal of domestic and industrial sewage are available. The concept of oxidation ponds in tropical countries has been found to be of utmost utility. By way of solar energy and its use, the sewage can be very well degraded and even has been found to be of reuse in aquaculture, agriculture and other uses.

In order to reuse the domestic wastewater or sewage for the fish culture, it requires that the sewage must be physically and biologically treated and this treatment of domestic wastewater for its purification gives rise to the concept of proper functioning of the oxidation or the stabilization ponds as suggested by several pioneer workers in the field, (Allen, 1970; Allen and Hepher, 1976 and Mara, 1976; Ali, 1988, 1991, 2000, and Ali et al., 2020, Prakash & Verma 2020; Shobana et al., 2021). The culture of fishes in highly nutrient waters has been made evident by the experiments of these above workers, which have demonstrated that fishes can grow considerably fast because of the easy and highly nutrient food web available to the fishes. It has become clearly known that fishes show significant changes in their growth rates.

## CONCLUSION

Domestic sewage has become a very attractive proposition for the recovery of valuable protein and its products it has been found to be quite rich in nutrients. Fish and other aquaculture products have grown in domestic sewage and offer excellent high-yield opportunities. Fishes grew in domestic sewage in developing and developed countries have been found to be quite suitable for human consumption. It is concluded that aquaculture is the best alternative way to remove eutrophication from water by taking the excessive amount of nutrients such as nitrogen and phosphorus by fishes that were produced after the oxidation of ponds and ultimately maintaining the water quality. This is the best alternative way and also plays an important role in removing the eutrophication from sewage for aquaculture. The biological treatment of domestic sewage oxidation ponds is one of the economical methods to produce fish and also can reduce the aquatic pollution. However, more work has to be carried out in this aspect to remove myths and misbelieves about sewage pond aquaculture.

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# Purification and Characterization of Keratinase from *A. aneurinilyticus* Isolated from Xerophytic Plant *Opuntia Ficus-Indica*

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## ABSTRACT

Keratinases from *Aneurinibacillus aneurinilyticus* are capable of degrading keratinous proteins. Purification and Characterization of the enzyme was carried out by salt precipitation, diethylaminoethyly, Ion-exchange and Gel permeation chromatography, and SDS-PAGE. Physicochemical factors like pH, temperature, metal ions, enzyme inhibitors and substrate. To study Km and Vmax various concentrations of keratin were used for the activity of enzyme. Gel permeation chromatography with 20.84-fold purification and 203.87 U/mg specific activity showing 34KDa between 14 to31KDa in SDS-PAGE, stable at pH 7.0-9.0 $40^0$-50 $^0$C, optimum at pH 9.0 and 50 $^0$C. Stimulated by $Mg^{2+}$, $Ca^{2+}$, $K^+$, $Fe^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Na^{2+}$ inhibited by $Cu^{2+}$, $Co^{2+}$ and $Hg^{2+}$. Ethylene diamine tetra acetic acid with the highest stimulatory effect, inhibited by Di-isopropyl fluoro phosphatase and phenyl methyl sulfonyl fluoride. Enzyme stable with Tween-60, TritonX-100 and TritonX-114 declined with ß-mercaptoethanol. It hydrolyzed several keratinous substrates as keratin and casein were 100 and 85.47% utilized with Km=3mM, Vmax =249µmol/ml/min. Xerophytic endophytes are treasure houses as they tolerate biotic and abiotic stress, are stable at high temperatures and pH are selected, such keratinases are used in leather processing and detergent industries.

**KEY WORDS:** ANEURINIBACILLUS ANEURINILYTICUS, CHARACTERIZATION, KERATINASE, KERATIN, PURIFICATION.

## INTRODUCTION

The insoluble keratin which is highly stable is present in fur, feathers, beak, horns, nails, and hair of living animals (Onifade et al. 1998; Pandian et al. 2012). Keratin fiber length depends on their water-containing complex hydration which increases their length by 10-12 % (Bhuyar et al. 2018). According to the sulfur content, hair, nail, hoof and also feathers are grouped as hard keratins, callus and skin belongs to soft keratins. Hard keratins from chicken feathers obtained from feather industries have high level of disulphide bonds, cross linked which are resistant and insoluble having hydrophobic interactions (Annapurna et al. 1996). Chicken feathers constitute 5-7% of keratin producing a large number of poultry wastes which decomposes slowly causing massive environmental concern (Ningthoujam et al. 2016; Almahasheer et al. 2022).

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Feather keratin characteristically attributes to the presence of various amino acids (Kumar et al. 2011; Gupta and Singh 2014). Physical and chemical methods of chicken feathers degradation consume high energy thus, damaging the products (Zoccola et al. 2012; Lee et al. 2016; Holkar et al. 2016). Hence, an alternative method of converting the keratinous wastes into economic, reusable, ecofriendly products by using such enzymes produced from vast microbial sources. Such enzymes are produced by various keratinolytic microorganisms. Keratinous substrates can be hydrolyzed by keratin degrading enzymes which have potential applications. keratinases are produced by Fungi, Bacteria and Actinomycetes (Onifade et al. 1998; Gupta and Ramnani 2006; Bhuyar et al. 2018; Almahasheer et al. 2022). Feather degradation by the enzyme keratinase produced by *B. subtilis, B. cereus,* and *B. licheniformis* has been reported (Thys et al. 2004; Almahasheer et al. 2022).

Keratinase are used in several biotechnological processes, such as removing hair and feather for the production of feather meal, clearing the obstructions in the sewage systems

and dehairing process in the leather industry (Gupta et al. 2002). Production of biogas, biodegradable films, glues, prion hydrolysis, wastewater processing, recovery of silver from X-ray films, drug delivery in medicine cosmetics and additives (Patinvoh et al. 2016; Almahasheer et al. 2022).

This work highlights the characterization and purification of the enzyme keratinase from *Aneurinibacillus aneurinilyticus* isolated from an endophytic xerophyte (*Opuntia-Ficus indica*). Lots of work has been done on keratinase production, purification and characterization by bacteria. Ours appears to be the first work on endophytic bacteria from xerophytes as they were isolated from drought regions, these enzymes from such bacteria can tolerate high temperatures and pH which can be used in various biotechnological industries. The keratin degrading protein i.e., keratinases has diversified application in green technology. Therefore, there is a search for an efficient bacterial strain by the researchers (Almahashree et al. 2022). The keratin from poultry waste is degraded by the purified enzyme keratinases from *B. licheniformis* dcs1, has the efficiency to enhance the nutritional quality of the waste keratin from poultry (Liaqat et al., 2022).

## MATERIAL AND METHODS

Bacteria was grown in 1000 ml production media under submerged fermentation and fermenting broth at 10,000 rpm at 4 $^0$C for 10 min. was centrifuged. Obtained supernatant was considered as a crude enzyme for purification purposes and at each step, enzyme activity was estimated. The crude enzyme from *A. aneurinilyticus* was partially purified further, ammonium sulphate precipitation was used at different saturation levels (10-80%) as per the standard chart with continuous stirring overnight under cold conditions to precipitate the desired protein.

Collected precipitate were centrifuged at 4 $^0$C for 10 min., further protein pellet were dissolved in 20 mmol/L Tris-HCl buffer (pH8.0). Folin-Phenol reagent along with Bovine Serum Albumin was used as standard for protein estimation. The protein sample was loaded into the activated dialysis membrane was sealed at both ends. The dialysis 150 membrane bag was suspended in 500mL (50 mM, pH 7.0) phosphate buffer was used. Experiment carried out with four changes in the buffer (Kamble et al. 2020).

The dialysate obtained from the above step was concentrated using (DEAE-cellulose), chromatographic anion coloumn (1.5X30cm). Sodium chloride with different concentration (pH-8.0) in 20mmol/L Tris HCl buffer were used to elute the sample at 6 ml/min flow rate for collecting the fractions (Cheng-Gang Cai et al. 2008). The concentrated enzyme was equilibrated with 100mM Tris-HCl buffer (pH8.0) and Sephadex G-100 (50cm X 1.7 Cm),gel filtration was used. The eluted pure protein fractions were characterized at the rate of 1ml/min of the fraction collected using same buffer. 10% separating acryl amide gel, 5% stacking gel having 0.1% SDS were used to visualize protein bands by Coomassei Brilliant Blue R-250 and destained with acetic acid and methanol. Such pure enzyme is used for characterization (Lammli 1970; Cheng-Gang Cai et al. 2008).

Bovine Serum Albumin 66KDa, Lysozyme 14.3kDa, Phosphorylase 97kDa, Ovalbumin 45kDa and Bovine Carbonic Anhydrase 31 kDa, were used as standard protein markers (Eun-Jan Jeong et al. 2010). Zymogram analysis was performed by using 2% keratin as substrate copolymerized with stacking gel of 4% and 10% resolving gel, the sample buffer and purified proteins together were loaded to polymerized gel. Tris-HCl buffer 0.01M having a pH of 9.0 Triton X-100 (v/v) 2.0% were subjected to electrophoresis, for about half an hour. To remove Triton X-100 the gel was washed with D. $H_2O$ and incubated at 37 $^0$C for 30 min. After incubating at pH-9.0 and 37 $^0$C for 30mins in Tris-Cl, stained with Coomassie Brilliant Blue Dye R-250 for half an hour then de-stained, clear colourless zone was detected and considered as enzyme activity (Vermelho et al. 2009). For the characterization of purified keratinase, various physicochemical factors like temperature, pH, metal ions, enzyme inhibitors along with substrate were studied.

Enzyme activity was noted using 0.1M acetate buffer (pH3-5), sodium phosphate buffer 0.1M (pH 6.0-8.0), glycine sodium hydroxide buffer (pH 9.0-12.0) with 0.1M Tris HCl buffer (pH 7.0-9.0) and temperature 10-80 $^0$C were taken to study the effect of pH and temperature on enzyme activity. The residual activities were measured by studying the effect of temperature and pH on enzyme stability by incubating the enzyme solution at pH between 3.0-12.0 and temperature between 10-80 $^0$C for 60 min (Murthy et al. 2019). The effect of enzyme inhibitors such as Ethylene Diamine Tetra Acetic Acid (EDTA), Phenyl Methyl Sulfonyl Fluoride (PMSF), Dithiothreitol (DTT), Diisopropyl Fluoro Phosphate (DFP), Sodium Dodecyl Sulfate (SDS), β-mercaptoethanol, Triton X-114, Triton X-100, Tween-60, at 5mM (1%) along with Purified keratinase were pre-incubated in 100mM Glycine /NaOH buffer for 1h. The enzyme without inhibitor serves as control and was taken as 100% activity (Ghasemi et al. 2012). Along with the effect of metal ions such as $Cu^{2+}$, $Co^{2+}$, $Hg^{2+}$, $Na^{2+}$, $K^{+}$, $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Zn^{2+}$(1mM each) then incubating the purified fraction to determine the effect of metal ions.

Different substrates such as haemoglobin, bovine serum albumin, fibrin, gelatin, keratin, and casein (1% w/v) were used for determining the enzyme Substrate specificity. The enzyme was incubated for10 min in each of the substrates. The degree of substrate hydrolysis was analyzed by protease activity using standard assay conditions (Gupta and Singh 2014). For measuring the enzyme activity various concentrations of keratin (1mM-10mM) dissolved in 0.05 M glycine NaOH buffer of pH 9.0 and incubation time of 30 min. under standard assay conditions were used for determining the Kinetic parameters Km, Vmax of purified keratinase by ploting Line weaver-Burk plot (1934).

## RESULT AND DISCUSSION

**Enzyme mass production:** Further the crude enzyme was used for purification steps.

**Purification of crude enzyme:** Salt precipitation, Dialysis, Gel filtration, SephadexG-100, DEAE cellulose were used for keratinase Purification from *A. aneurinilyticus*. Over all it was observed that the specific activity was 203.87U/mg, yield of 25.43% with a fold increase of 20.84 (Table-1).

Keratinases from *P. vulgaris* EMB-14 showed purification folds of 14.46 with specific activity of 74.74 U/mg and 8.27% yield as reported by (Dada et al. 2020). A 10-fold purification with 3.46% yield and specific activity of 297.5U/mg with recovery of 45% was and keratinase with 7.5-fold increases in a specific activity with 45% recovery was reported in the previous studies (Ire et al. 2017). Keratinase with a 7.5-fold increase in a specific activity with 45% recovery (Adina et al. 2021). A similar result was observed with a single band at 26KDa both in zymogram and SDS PAGE with *B. licheniformis* H62 (Kazzaz et al., 2015). *B. subtilis* SCK6 showed 30.95KDa and *B. amyloliquefaciens* strain TCCC 11319 with 28KDa (Tian et al. 2019), Zhang et al. (2016) Han et al. (2012) also reported a nearer mol. wt. of 25KDa and 33KDa of keratinase from *P. aeruginosa* C11. The reports of various authors producing keratinase from *Bacillus* sps. ,correlates with our results (Tian et al. 2019; Adina et al. 2021).

**Table 1. Steps of keratinase Purification from *A.aneurinilyticus*VRCS-4**

| Steps of Purification | Activity (U/ml) | Protein (mg) | Specific activity (U/mg) | Fold of Purification | (%) Yield |
|---|---|---|---|---|---|
| Crude culture fluid $(NH_4)_2SO_4$ | 38478 | 3931 | 9.78 | 1.0 | 100 |
| Precipitation (70%) | 27863 | 2453 | 11.35 | 1.16 | 74.41 |
| DEAE cellulose | 11602 | 143 | 81.13 | 8.29 | 30.15 |
| CM Sephadex G-100 Gel filtration | 9786 | 48 | 203.87 | 20.84 | 25.43 |

**Figure 1.a: SDS PAGE of keratinase (M: Marker protein), (1: Keratinase).**
**Figure 1.b: Keratinase Zymogram**

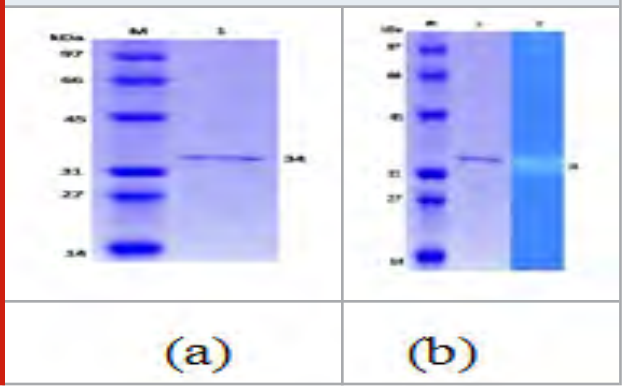



**Figure: 2 Influence of pH on keratinase activity**

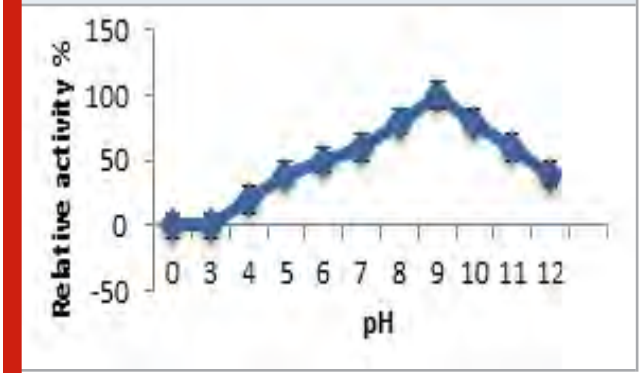
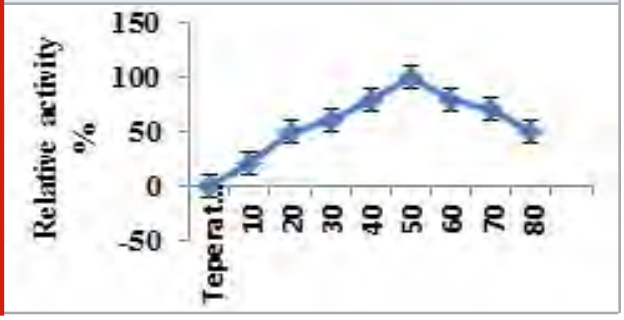



**Figure 3: Influence of temperature on keratinase activity**




Keratinase from *A. aneurinilyticus* and its molecular weight were determined by comparing the electrophoretic mobility of the marker proteins. Standard protein markers used were 97KDa (phosphorylase), 66KDa (BSA), 45KDa (Ovalbumin), and 31KDa (Bovine carbonic anhydrase) and 14.33KDa (Lysozyme). The mol. wt. of the purified enzyme was 34KDa when SDS PAGE was done (Fig.1a). Zymogram analysis showed a clear colourless zone against a dark blue background (Fig.1b). Similar results were observed with Kazzaz et al. (2015) revealing a single band at 26KDa both in zymogram and SDS PAGE with *B. licheniformis* H62. *B. subtilis* SCK6 showed 30.95KDa and *B. amyloliquefaciens* strain TCCC 11319 with 28KDa (Tian et al. 2019, Zhang et al., 2016) and Han et al., (2012) also reported almost nearer mol. wt. of 25KDa and 33KDa of keratinase from *P. aeruginosa* C-11. Liaqat et al. (2022) also purified and characterized keratinase from *B. licheniformis* dcs1 from poultry waste processing and the keratinase which correlates our results, keratinase with mol. wt. of 34KDa. as reported

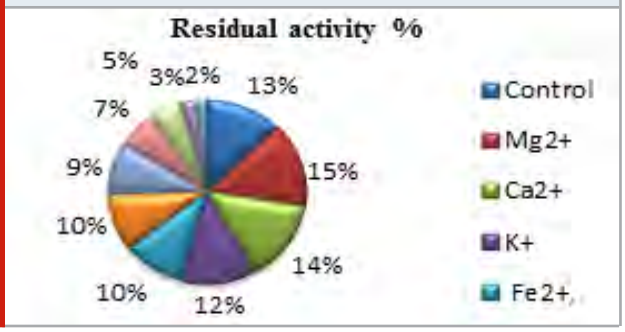
by (Yong et al. 2020). Thus, showing similar reports of keratinase production with almost similar mol.Wt. from *Bacillus* sps (Yong et al. 2020; Liaqat et al. 2022).

**Characterization of Purified Enzyme:** Maximum activity of purified keratinase enzyme was at 8.0 to10.0 pH, while moderate activity was between 5.0-11.0. Relative keratinase activity which was maximum (100%) was at pH 9.0 (Fig.2). To check the enzyme stability from *A. aneurinilyticus* it was pre incubated between the temperatures of 20-80 $^0$C. It was observed that the enzyme was stable at 50 $^0$C. Further, increase in the temperature to 80 $^0$C, the relative activity was reduced to 40% (Fig.3) by increasing the temperature to 80 $^0$C, revealing that the purified keratinase showed the optimum activity at pH9.0and temperature 50$^0$C (Kumar et al. 2021).

Similar results of optimum pH and temperature with *B. megaterium* were reported by Saibabu et al. (2012). Using the organism *Chrysosporium indicum* as reported by Kumar et al. (2021) it also showed maximum keratinase activity at temperature 50 $^0$C and pH-10. Yong et al. (2020) reported optimal temperature, pH 55 $^0$C and 10.0 by *B. subtilis* S1-4. When these results of keratinase production were compared with all the researchers it was observed that the pH ranged between 8.0 -9.0 and the temperature between 50-60 $^0$C (Kumar et al. 2021).

Keratinase activity on metal ions is presented (Fig. 4). The metal ions, studied were $Mg^{2+}$, $Ca^{2+}$, $K^{+}$, $Fe^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Na^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Co^{2+}$ at 1mM concentration. The percent residual activity was maximum with $Mg^{2+}$, $Ca^{2+}$, $K^{+}$, $Fe^{2+}$, $Zn^{2+}$and $Mn^{2+}$ while $Cu^{2+}$, $Co^{2+}$, and $Hg^{2+}$ showed inhibitory action against keratinase from *A. aneurinilyticus.* Keratinase activity was inhibited by $Hg^{2+}$, which signifies that $Hg^{2+}$, might reduce the enzyme activity by binding to the SH-group present at the active site or $Hg^{2+}$ may bind to the carboxylic group and also tryptophan residue to decrease the enzyme activity (Saibu et al. 2013; Ul–Haq 2020). While in case with *A. aneurinilyticus* some cations stimulated the keratinase activity. Saibabu et al. (2013) reported that the enzyme inhibition with $Hg^{2+}$ is not due to the thiol group but is due to the tryptophan residue interaction or may be the carboxyl group present in the amino acid of that particular enzyme. The stimulatory effect of $Fe^{2+}$, $Ca^{2+}$and $Mn^{2+}$ at 1mM increases the keratinase activity by acting as co-factors (Ul–Haq 2020).

**Figure 4: Effect of metal ions on keratinase activity**




Suntornsuk et al. (2005) reported that $Ca^{2+}$ a divalent cat ions enhanced the enzyme activity of *B. licheniformis* FK-14, indicating the enzyme to be typical for serine protease. To maintain the enzyme structural confirmation or to stabilize the substrate binding and to form enzyme complex probably these metal ions may be acting as ion bridge. Keratinase from *A. aneurinilyticus* is highly activated by $Mg^{2+}$, $Ca^{2+}$ and $Mn^{2+}$ proving that these metal ions confer for protection of enzyme against denaturation by heat performing a vital function in the maintenance of its active conformation (Kumar and Takagi 1999). Dada (2020) expressed that keratinase largely from gram-positive bacteria are mostly serine proteases as they possess two $Ca^{2+}$ active sites thereby enhancing the enzymatic activity. The role of $Ca^{2+}$ associated with the stability of the activated forms of the keratinases agrees with our results (Dada 2020; Ul–Haq 2020).

**Figure 5: Effect of enzyme inhibitors on keratinase activity**

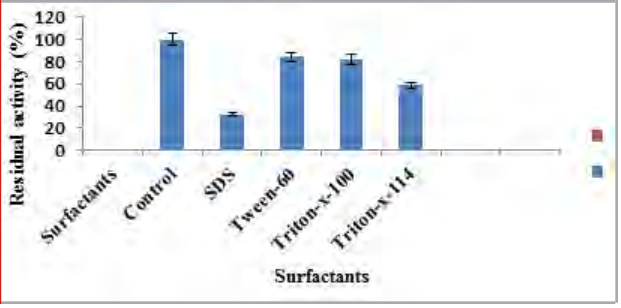
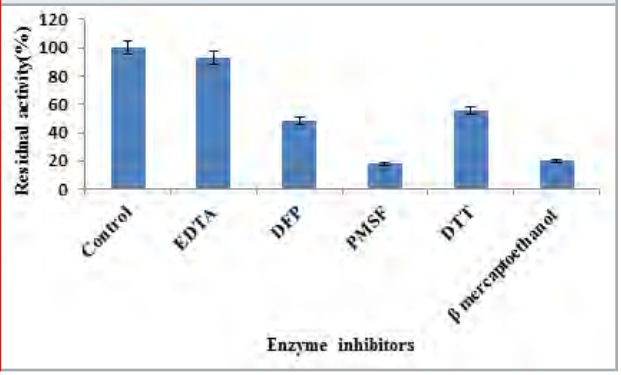



**Figure 6: Effect of surfactant on enzyme activity**




Keratinase was stimulated by EDTA, DTT, DFP while the most potent inhibitor was PMSF (Fig.5). When the surfactants results were compared the keratinase activity was inhibited (19.92%) to the maximum with β-mercaptoethanol and stimulated by tween-60 (84.11%), tritonX-100 (82.01%), tritonX-114 (58.65%) and SDS (32.46%) (Fig.6). Inhibition of the enzyme activity in presence of PMSF indicates the keratin belongs to serine group. This inhibition is due to covalent binding of PMSF to the residual serine and in activating it, there by blocking the sulphonate releasing hydrolytic sides for keratinolytic attack (Suntornsuk et al. 2005). The active site of protease was blocked by PMSF by sulfonating the essential serine residue, there by resulting in complete inhibition of protease

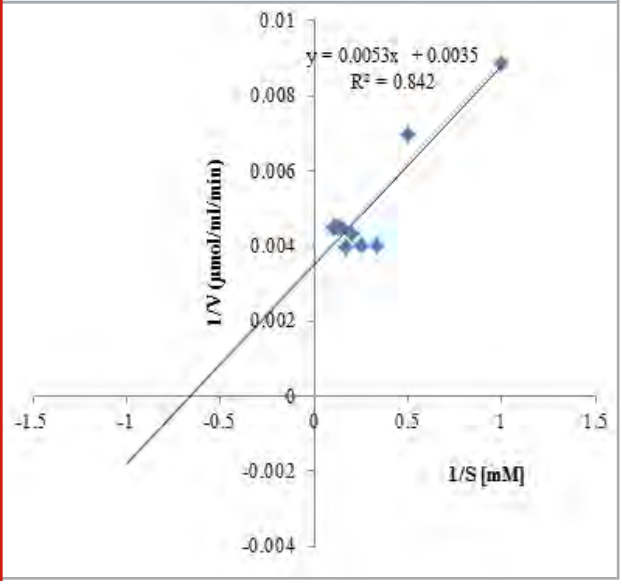
activity (Jaouadi et al. 2013). However,reducing agents able to break the disulphide bonds in the substrate keratin releasing different hydrolytic sites for the keratinolytic attack (Dada 2020; Ul–Haq 2020).

Moreover 70% of reduction in enzyme activity was observed with β-mercaptoethanol. Xian et al. (2016) also presented 70% of enzyme inhibition with β-mercaptoethanol, while it was 55% with DTT. More than 50% of residual activity was with other nonionic surfactants. We evaluated the impact of inhibitors and surfactants on keratinase activity by *A. aneurunilyticus*. Enzyme activity was inhibited by PMSF, β-mercaptoethanol and stimulated by EDTA, while amongst the surfactant used is again stimulated by Tween-60 and anionic surfactant such as SDS inhibited them. Our results correlate with Bose et al. (2014) where PMSF completely inhibited the enzyme activity while nonionic and anionic were marginal stable. Stability of the keratinase enzyme from *B. subtilis* k-5 with SDS and Tween-80 while activity was inhibited by β-mercaptoethanol was reported by Singh et al. (2014). Rai and Mukharjee (2009) stated that the stable alkaline protease in presence of surfactants is highly desirable for industrial application (Ul–Haq 2020).

**Table 2. Specificity of keratinase in the presence of different substrate**

| Substrates | Enzyme activity (U/ml) | Relative activity (%) |
|---|---|---|
| Bovine serum albumin | 252.5 | 69.04 |
| Casein | 312.0 | 85.47 |
| Gelatin | 218.0 | 59.72 |
| Fibrin | 136.0 | 36.43 |
| Keratin | 365.0 | 100.00 |
| Haemoglobin | 176.0 | 48.21 |

**Figure 7: Line Weaver-Burk plot (1/[S] V/S 1/ [V]) Km and Vmax**




Keratinases from *A. aneurunilyticus* showed broad substrate specificity since it could hydrolyze keratin substrate and casein (85.47%), BSA (69.04%), gelatin (59.72%), haemoglobin (48.21%) and the least were fibrin (36.43%) (Table-2). Moridshashi et al. (2020) reported maximum relative activity with feather keratin then was casein, and the least hydrolysed were keratin azure, gelatine and BSA. Prakash et al. (2010) reported high activity towards casein, followed by keratin, using *B. halodurans* PPKS-2. Gang et al. (2008) also revealed maximum keratinase activity by using casein, BSA, Feather meal and feather keratin with the organism *B. subtilis* KD-N2. When the relative activity of different substrates was compared it was observed that the substrates which were having more disulphide bonds could be easily hydrolysed than the substrates having less disulphide bonds, suggesting the solubility depends on the high percentage of disulphide bonds in the substrates (Moridshashi et al. 2020).

Different concentrations of the substrate keratin (1mM to10mM) were used to determine the Km and Vmax of purified keratinase. Km and Vmax values were determined by LB plot and were found to be 3mM-Km and 249µmol/mL/min Vmax respectively (Fig.7). Lower values of Km suggest high affinity towards the substrates indicating that the enzyme-substrate complex is tightly held before the substrate is converted to the product thus indicating that keratinase enzyme from *A. aneurinilyticus* has great affinity towards its substrate keratin. 1/S on the X-axis, 1/V on the Y-axis, a double reciprocal plot gave a straight line suggesting that our enzyme obeys the Michaelis Menton equation. Km being independent of enzyme concentration shows the characteristics of enzyme under defined temperature and pH condition. (Moridshashi et al. 2020).

Singh (2014) and Srinivasan (2008) also reported a low Km value of 0.01µg/ml/min and Vmax of 1176mg/ml with *B. subtillis* K-1. Dada (2020) also reported Km and Vmax kinetic constants of 25.60mM and 74.46U/ml respectively, with *B. licheniformis* K-51. Hydrolysis efficiency of 7mg/ml for Km and 384.6U/mg of Vmax was observed when casein was the substrate, while it was 7.2mg/ml and 103mg/ml respectively when the substrate was keratin azure with *Laeeyella sacchari* (Dada et al. 2020). Moridshashi et al. (2020) also reported a low Km value of 8.74mg/ml and Vmax of 59.04U/ml/min with feather meal substrate using *B. zhangzhouensis*. The above-said report correlates with ours (Dada et al. 2020).

## CONCLUSION

The findings of the present study showed that proteases of microbial origin are interesting, compared to plants or animal sources, as these enzymes from microbial origin possess all the features desired for biotechnological applications. Now, microorganisms are considered as efficient feather degraders. Consequently, keratin can be transformed by keratinolytic microorganisms such as *A. aneuinilyticus* with low molecular weight of (34KDa). Generally, the molecular weight of bacterial keratinases varied between species and combination of various peptidases is required for keratin degradation. Thus, keratinase with low mol. wt. can be

used in various biotechnological processes. Our studies of keratinase production from xerophytic endophytes will unravel the complex mechanism of keratinolysis and stability of the enzymes at high pH and temperatures which can be employed in various industries.

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**Conflict of interests:** Authors declare no conflict of interests to disclose.

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# Purification and Characterization of Keratinase from *A. aneurinilyticus* Isolated from Xerophytic Plant *Opuntia Ficus-Indica*

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## ABSTRACT

Keratinases from *Aneurinibacillus aneurinilyticus* are capable of degrading keratinous proteins. Purification and Characterization of the enzyme was carried out by salt precipitation, diethylaminoethyly, Ion-exchange and Gel permeation chromatography, and SDS-PAGE. Physicochemical factors like pH, temperature, metal ions, enzyme inhibitors and substrate. To study Km and Vmax various concentrations of keratin were used for the activity of enzyme. Gel permeation chromatography with 20.84-fold purification and 203.87 U/mg specific activity showing 34KDa between 14 to31KDa in SDS-PAGE, stable at pH 7.0-9.0 $40^0$-50 $^0$C, optimum at pH 9.0 and 50 $^0$C. Stimulated by $Mg^{2+}$, $Ca^{2+}$, $K^+$, $Fe^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Na^{2+}$ inhibited by $Cu^{2+}$, $Co^{2+}$ and $Hg^{2+}$. Ethylene diamine tetra acetic acid with the highest stimulatory effect, inhibited by Di-isopropyl fluoro phosphatase and phenyl methyl sulfonyl fluoride. Enzyme stable with Tween-60, TritonX-100 and TritonX-114 declined with ß-mercaptoethanol. It hydrolyzed several keratinous substrates as keratin and casein were 100 and 85.47% utilized with Km=3mM, Vmax =249µmol/ml/min. Xerophytic endophytes are treasure houses as they tolerate biotic and abiotic stress, are stable at high temperatures and pH are selected, such keratinases are used in leather processing and detergent industries.

**KEY WORDS:** ANEURINIBACILLUS ANEURINILYTICUS, CHARACTERIZATION, KERATINASE, KERATIN, PURIFICATION.

## INTRODUCTION

The insoluble keratin which is highly stable is present in fur, feathers, beak, horns, nails, and hair of living animals (Onifade et al. 1998; Pandian et al. 2012). Keratin fiber length depends on their water-containing complex hydration which increases their length by 10-12 % (Bhuyar et al. 2018). According to the sulfur content, hair, nail, hoof and also feathers are grouped as hard keratins, callus and skin belongs to soft keratins. Hard keratins from chicken feathers obtained from feather industries have high level of disulphide bonds, cross linked which are resistant and insoluble having hydrophobic interactions (Annapurna et al. 1996). Chicken feathers constitute 5-7% of keratin producing a large number of poultry wastes which decomposes slowly causing massive environmental concern (Ningthoujam et al. 2016; Almahasheer et al. 2022).

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Feather keratin characteristically attributes to the presence of various amino acids (Kumar et al. 2011; Gupta and Singh 2014). Physical and chemical methods of chicken feathers degradation consume high energy thus, damaging the products (Zoccola et al. 2012; Lee et al. 2016; Holkar et al. 2016). Hence, an alternative method of converting the keratinous wastes into economic, reusable, ecofriendly products by using such enzymes produced from vast microbial sources. Such enzymes are produced by various keratinolytic microorganisms. Keratinous substrates can be hydrolyzed by keratin degrading enzymes which have potential applications. keratinases are produced by Fungi, Bacteria and Actinomycetes (Onifade et al. 1998; Gupta and Ramnani 2006; Bhuyar et al. 2018; Almahasheer et al. 2022). Feather degradation by the enzyme keratinase produced by *B. subtilis, B. cereus,* and *B. licheniformis* has been reported (Thys et al. 2004; Almahasheer et al. 2022).

Keratinase are used in several biotechnological processes, such as removing hair and feather for the production of feather meal, clearing the obstructions in the sewage systems

and dehairing process in the leather industry (Gupta et al. 2002). Production of biogas, biodegradable films, glues, prion hydrolysis, wastewater processing, recovery of silver from X-ray films, drug delivery in medicine cosmetics and additives (Patinvoh et al. 2016; Almahasheer et al. 2022).

This work highlights the characterization and purification of the enzyme keratinase from *Aneurinibacillus aneurinilyticus* isolated from an endophytic xerophyte (*Opuntia-Ficus indica*). Lots of work has been done on keratinase production, purification and characterization by bacteria. Ours appears to be the first work on endophytic bacteria from xerophytes as they were isolated from drought regions, these enzymes from such bacteria can tolerate high temperatures and pH which can be used in various biotechnological industries. The keratin degrading protein i.e., keratinases has diversified application in green technology. Therefore, there is a search for an efficient bacterial strain by the researchers (Almahashree et al. 2022). The keratin from poultry waste is degraded by the purified enzyme keratinases from *B. licheniformis* dcs1, has the efficiency to enhance the nutritional quality of the waste keratin from poultry (Liaqat et al., 2022).

## MATERIAL AND METHODS

Bacteria was grown in 1000 ml production media under submerged fermentation and fermenting broth at 10,000 rpm at 4 $^0$C for 10 min. was centrifuged. Obtained supernatant was considered as a crude enzyme for purification purposes and at each step, enzyme activity was estimated. The crude enzyme from *A. aneurinilyticus* was partially purified further, ammonium sulphate precipitation was used at different saturation levels (10-80%) as per the standard chart with continuous stirring overnight under cold conditions to precipitate the desired protein.

Collected precipitate were centrifuged at 4 $^0$C for 10 min., further protein pellet were dissolved in 20 mmol/L Tris-HCl buffer (pH8.0). Folin-Phenol reagent along with Bovine Serum Albumin was used as standard for protein estimation. The protein sample was loaded into the activated dialysis membrane was sealed at both ends. The dialysis 150 membrane bag was suspended in 500mL (50 mM, pH 7.0) phosphate buffer was used. Experiment carried out with four changes in the buffer (Kamble et al. 2020).

The dialysate obtained from the above step was concentrated using (DEAE-cellulose), chromatographic anion coloumn (1.5X30cm). Sodium chloride with different concentration (pH-8.0) in 20mmol/L Tris HCl buffer were used to elute the sample at 6 ml/min flow rate for collecting the fractions (Cheng-Gang Cai et al. 2008). The concentrated enzyme was equilibrated with 100mM Tris-HCl buffer (pH8.0) and Sephadex G-100 (50cm X 1.7 Cm),gel filtration was used. The eluted pure protein fractions were characterized at the rate of 1ml/min of the fraction collected using same buffer. 10% separating acryl amide gel, 5% stacking gel having 0.1% SDS were used to visualize protein bands by Coomassei Brilliant Blue R-250 and destained with acetic acid and methanol. Such pure enzyme is used for characterization (Lammli 1970; Cheng-Gang Cai et al. 2008).

Bovine Serum Albumin 66KDa, Lysozyme 14.3kDa, Phosphorylase 97kDa, Ovalbumin 45kDa and Bovine Carbonic Anhydrase 31 kDa, were used as standard protein markers (Eun-Jan Jeong et al. 2010). Zymogram analysis was performed by using 2% keratin as substrate copolymerized with stacking gel of 4% and 10% resolving gel, the sample buffer and purified proteins together were loaded to polymerized gel. Tris-HCl buffer 0.01M having a pH of 9.0 Triton X-100 (v/v) 2.0% were subjected to electrophoresis, for about half an hour. To remove Triton X-100 the gel was washed with D. $H_2O$ and incubated at 37 $^0$C for 30 min. After incubating at pH-9.0 and 37 $^0$C for 30mins in Tris-Cl, stained with Coomassie Brilliant Blue Dye R-250 for half an hour then de-stained, clear colourless zone was detected and considered as enzyme activity (Vermelho et al. 2009). For the characterization of purified keratinase, various physicochemical factors like temperature, pH, metal ions, enzyme inhibitors along with substrate were studied.

Enzyme activity was noted using 0.1M acetate buffer (pH3-5), sodium phosphate buffer 0.1M (pH 6.0-8.0), glycine sodium hydroxide buffer (pH 9.0-12.0) with 0.1M Tris HCl buffer (pH 7.0-9.0) and temperature 10-80 $^0$C were taken to study the effect of pH and temperature on enzyme activity. The residual activities were measured by studying the effect of temperature and pH on enzyme stability by incubating the enzyme solution at pH between 3.0-12.0 and temperature between 10-80 $^0$C for 60 min (Murthy et al. 2019). The effect of enzyme inhibitors such as Ethylene Diamine Tetra Acetic Acid (EDTA), Phenyl Methyl Sulfonyl Fluoride (PMSF), Dithiothreitol (DTT), Diisopropyl Fluoro Phosphate (DFP), Sodium Dodecyl Sulfate (SDS), β-mercaptoethanol, Triton X-114, Triton X-100, Tween-60, at 5mM (1%) along with Purified keratinase were pre-incubated in 100mM Glycine /NaOH buffer for 1h. The enzyme without inhibitor serves as control and was taken as 100% activity (Ghasemi et al. 2012). Along with the effect of metal ions such as $Cu^{2+}$, $Co^{2+}$, $Hg^{2+}$, $Na^{2+}$, $K^{+}$, $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Zn^{2+}$(1mM each) then incubating the purified fraction to determine the effect of metal ions.

Different substrates such as haemoglobin, bovine serum albumin, fibrin, gelatin, keratin, and casein (1% w/v) were used for determining the enzyme Substrate specificity. The enzyme was incubated for10 min in each of the substrates. The degree of substrate hydrolysis was analyzed by protease activity using standard assay conditions (Gupta and Singh 2014). For measuring the enzyme activity various concentrations of keratin (1mM-10mM) dissolved in 0.05 M glycine NaOH buffer of pH 9.0 and incubation time of 30 min. under standard assay conditions were used for determining the Kinetic parameters Km, Vmax of purified keratinase by ploting Line weaver-Burk plot (1934).

## RESULT AND DISCUSSION

**Enzyme mass production:** Further the crude enzyme was used for purification steps.

**Purification of crude enzyme:** Salt precipitation, Dialysis, Gel filtration, SephadexG-100, DEAE cellulose were used for keratinase Purification from *A. aneurinilyticus*. Over all it was observed that the specific activity was 203.87U/mg, yield of 25.43% with a fold increase of 20.84 (Table-1).

Keratinases from *P. vulgaris* EMB-14 showed purification folds of 14.46 with specific activity of 74.74 U/mg and 8.27% yield as reported by (Dada et al. 2020). A 10-fold purification with 3.46% yield and specific activity of 297.5U/mg with recovery of 45% was and keratinase with 7.5-fold increases in a specific activity with 45% recovery was reported in the previous studies (Ire et al. 2017). Keratinase with a 7.5-fold increase in a specific activity with 45% recovery (Adina et al. 2021). A similar result was observed with a single band at 26KDa both in zymogram and SDS PAGE with *B. licheniformis* H62 (Kazzaz et al., 2015). *B. subtilis* SCK6 showed 30.95KDa and *B. amyloliquefaciens* strain TCCC 11319 with 28KDa (Tian et al. 2019), Zhang et al. (2016) Han et al. (2012) also reported a nearer mol. wt. of 25KDa and 33KDa of keratinase from *P. aeruginosa* C11. The reports of various authors producing keratinase from *Bacillus* sps. ,correlates with our results (Tian et al. 2019; Adina et al. 2021).

**Table 1. Steps of keratinase Purification from *A.aneurinilyticus*VRCS-4**

| Steps of Purification | Activity (U/ml) | Protein (mg) | Specific activity (U/mg) | Fold of Purification | (%) Yield |
|---|---|---|---|---|---|
| Crude culture fluid $(NH_4)_2SO_4$ | 38478 | 3931 | 9.78 | 1.0 | 100 |
| Precipitation (70%) | 27863 | 2453 | 11.35 | 1.16 | 74.41 |
| DEAE cellulose | 11602 | 143 | 81.13 | 8.29 | 30.15 |
| CM Sephadex G-100 Gel filtration | 9786 | 48 | 203.87 | 20.84 | 25.43 |

**Figure 1.a: SDS PAGE of keratinase (M: Marker protein), (1: Keratinase).**
**Figure 1.b: Keratinase Zymogram**

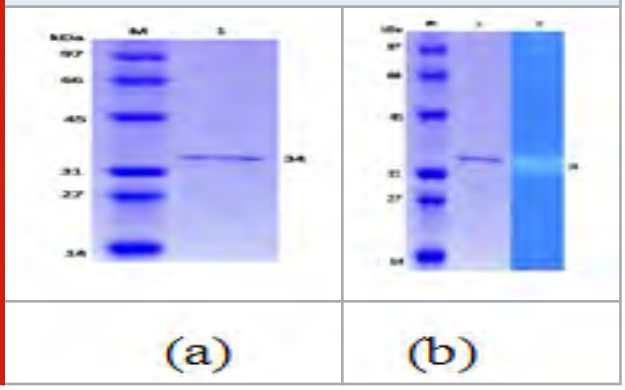



**Figure: 2 Influence of pH on keratinase activity**

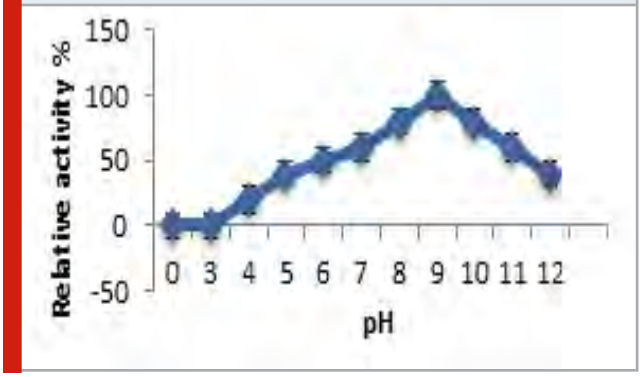
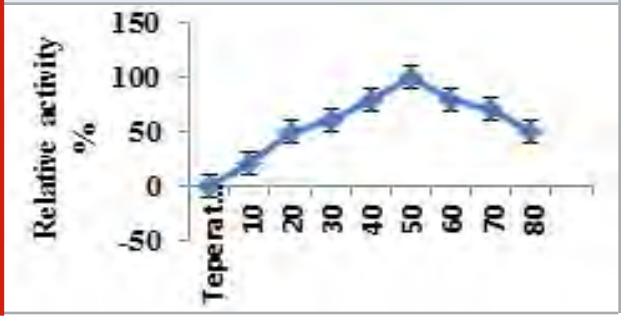



**Figure 3: Influence of temperature on keratinase activity**




Keratinase from *A. aneurinilyticus* and its molecular weight were determined by comparing the electrophoretic mobility of the marker proteins. Standard protein markers used were 97KDa (phosphorylase), 66KDa (BSA), 45KDa (Ovalbumin), and 31KDa (Bovine carbonic anhydrase) and 14.33KDa (Lysozyme). The mol. wt. of the purified enzyme was 34KDa when SDS PAGE was done (Fig.1a). Zymogram analysis showed a clear colourless zone against a dark blue background (Fig.1b). Similar results were observed with Kazzaz et al. (2015) revealing a single band at 26KDa both in zymogram and SDS PAGE with *B. licheniformis* H62. *B. subtilis* SCK6 showed 30.95KDa and *B. amyloliquefaciens* strain TCCC 11319 with 28KDa (Tian et al. 2019, Zhang et al., 2016) and Han et al., (2012) also reported almost nearer mol. wt. of 25KDa and 33KDa of keratinase from *P. aeruginosa* C-11. Liaqat et al. (2022) also purified and characterized keratinase from *B. licheniformis* dcs1 from poultry waste processing and the keratinase which correlates our results, keratinase with mol. wt. of 34KDa. as reported

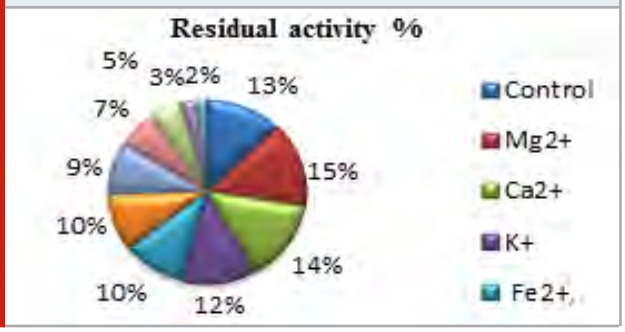
by (Yong et al. 2020). Thus, showing similar reports of keratinase production with almost similar mol.Wt. from *Bacillus* sps (Yong et al. 2020; Liaqat et al. 2022).

**Characterization of Purified Enzyme:** Maximum activity of purified keratinase enzyme was at 8.0 to10.0 pH, while moderate activity was between 5.0-11.0. Relative keratinase activity which was maximum (100%) was at pH 9.0 (Fig.2). To check the enzyme stability from *A. aneurinilyticus* it was pre incubated between the temperatures of 20-80 $^0$C. It was observed that the enzyme was stable at 50 $^0$C. Further, increase in the temperature to 80 $^0$C, the relative activity was reduced to 40% (Fig.3) by increasing the temperature to 80 $^0$C, revealing that the purified keratinase showed the optimum activity at pH9.0and temperature 50$^0$C (Kumar et al. 2021).

Similar results of optimum pH and temperature with *B. megaterium* were reported by Saibabu et al. (2012). Using the organism *Chrysosporium indicum* as reported by Kumar et al. (2021) it also showed maximum keratinase activity at temperature 50 $^0$C and pH-10. Yong et al. (2020) reported optimal temperature, pH 55 $^0$C and 10.0 by *B. subtilis* S1-4. When these results of keratinase production were compared with all the researchers it was observed that the pH ranged between 8.0 -9.0 and the temperature between 50-60 $^0$C (Kumar et al. 2021).

Keratinase activity on metal ions is presented (Fig. 4). The metal ions, studied were $Mg^{2+}$, $Ca^{2+}$, $K^{+}$, $Fe^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Na^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Co^{2+}$ at 1mM concentration. The percent residual activity was maximum with $Mg^{2+}$, $Ca^{2+}$, $K^{+}$, $Fe^{2+}$, $Zn^{2+}$and $Mn^{2+}$ while $Cu^{2+}$, $Co^{2+}$, and $Hg^{2+}$ showed inhibitory action against keratinase from *A. aneurinilyticus.* Keratinase activity was inhibited by $Hg^{2+}$, which signifies that $Hg^{2+}$, might reduce the enzyme activity by binding to the SH-group present at the active site or $Hg^{2+}$ may bind to the carboxylic group and also tryptophan residue to decrease the enzyme activity (Saibu et al. 2013; Ul–Haq 2020). While in case with *A. aneurinilyticus* some cations stimulated the keratinase activity. Saibabu et al. (2013) reported that the enzyme inhibition with $Hg^{2+}$ is not due to the thiol group but is due to the tryptophan residue interaction or may be the carboxyl group present in the amino acid of that particular enzyme. The stimulatory effect of $Fe^{2+}$, $Ca^{2+}$and $Mn^{2+}$ at 1mM increases the keratinase activity by acting as co-factors (Ul–Haq 2020).

**Figure 4: Effect of metal ions on keratinase activity**




Suntornsuk et al. (2005) reported that $Ca^{2+}$ a divalent cat ions enhanced the enzyme activity of *B. licheniformis* FK-14, indicating the enzyme to be typical for serine protease. To maintain the enzyme structural confirmation or to stabilize the substrate binding and to form enzyme complex probably these metal ions may be acting as ion bridge. Keratinase from *A. aneurinilyticus* is highly activated by $Mg^{2+}$, $Ca^{2+}$ and $Mn^{2+}$ proving that these metal ions confer for protection of enzyme against denaturation by heat performing a vital function in the maintenance of its active conformation (Kumar and Takagi 1999). Dada (2020) expressed that keratinase largely from gram-positive bacteria are mostly serine proteases as they possess two $Ca^{2+}$ active sites thereby enhancing the enzymatic activity. The role of $Ca^{2+}$ associated with the stability of the activated forms of the keratinases agrees with our results (Dada 2020; Ul–Haq 2020).

**Figure 5: Effect of enzyme inhibitors on keratinase activity**

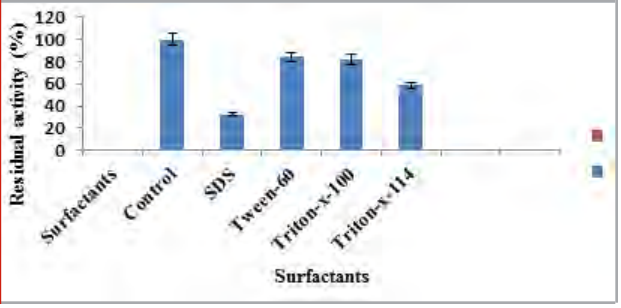
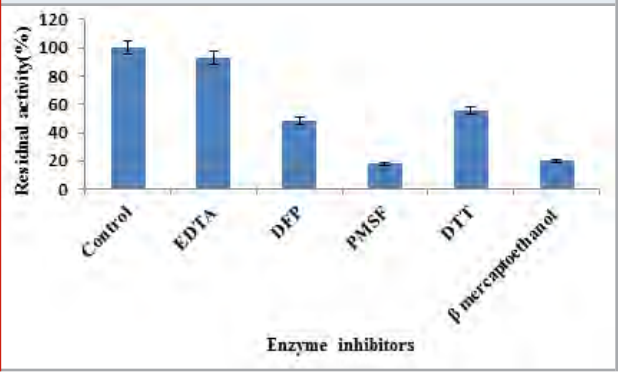



**Figure 6: Effect of surfactant on enzyme activity**




Keratinase was stimulated by EDTA, DTT, DFP while the most potent inhibitor was PMSF (Fig.5). When the surfactants results were compared the keratinase activity was inhibited (19.92%) to the maximum with β-mercaptoethanol and stimulated by tween-60 (84.11%), tritonX-100 (82.01%), tritonX-114 (58.65%) and SDS (32.46%) (Fig.6). Inhibition of the enzyme activity in presence of PMSF indicates the keratin belongs to serine group. This inhibition is due to covalent binding of PMSF to the residual serine and in activating it, there by blocking the sulphonate releasing hydrolytic sides for keratinolytic attack (Suntornsuk et al. 2005). The active site of protease was blocked by PMSF by sulfonating the essential serine residue, there by resulting in complete inhibition of protease

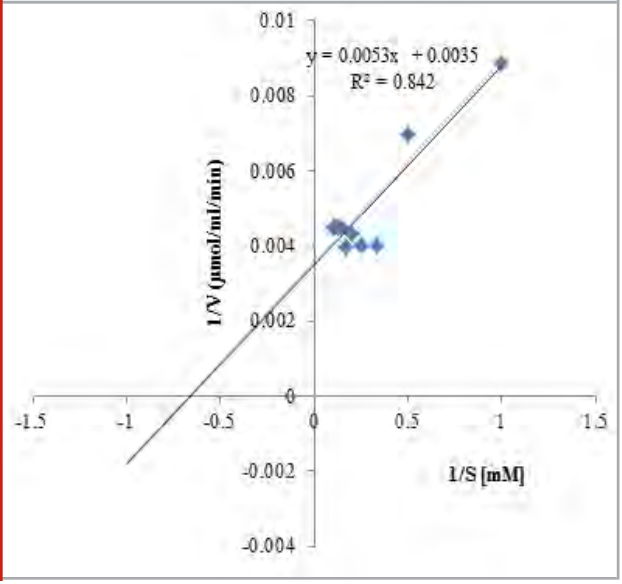
activity (Jaouadi et al. 2013). However,reducing agents able to break the disulphide bonds in the substrate keratin releasing different hydrolytic sites for the keratinolytic attack (Dada 2020; Ul–Haq 2020).

Moreover 70% of reduction in enzyme activity was observed with β-mercaptoethanol. Xian et al. (2016) also presented 70% of enzyme inhibition with β-mercaptoethanol, while it was 55% with DTT. More than 50% of residual activity was with other nonionic surfactants. We evaluated the impact of inhibitors and surfactants on keratinase activity by *A. aneurunilyticus*. Enzyme activity was inhibited by PMSF, β-mercaptoethanol and stimulated by EDTA, while amongst the surfactant used is again stimulated by Tween-60 and anionic surfactant such as SDS inhibited them. Our results correlate with Bose et al. (2014) where PMSF completely inhibited the enzyme activity while nonionic and anionic were marginal stable. Stability of the keratinase enzyme from *B. subtilis* k-5 with SDS and Tween-80 while activity was inhibited by β-mercaptoethanol was reported by Singh et al. (2014). Rai and Mukharjee (2009) stated that the stable alkaline protease in presence of surfactants is highly desirable for industrial application (Ul–Haq 2020).

**Table 2. Specificity of keratinase in the presence of different substrate**

| Substrates | Enzyme activity (U/ml) | Relative activity (%) |
|---|---|---|
| Bovine serum albumin | 252.5 | 69.04 |
| Casein | 312.0 | 85.47 |
| Gelatin | 218.0 | 59.72 |
| Fibrin | 136.0 | 36.43 |
| Keratin | 365.0 | 100.00 |
| Haemoglobin | 176.0 | 48.21 |

**Figure 7: Line Weaver-Burk plot (1/[S] V/S 1/ [V]) Km and Vmax**




Keratinases from *A. aneurunilyticus* showed broad substrate specificity since it could hydrolyze keratin substrate and casein (85.47%), BSA (69.04%), gelatin (59.72%), haemoglobin (48.21%) and the least were fibrin (36.43%) (Table-2). Moridshashi et al. (2020) reported maximum relative activity with feather keratin then was casein, and the least hydrolysed were keratin azure, gelatine and BSA. Prakash et al. (2010) reported high activity towards casein, followed by keratin, using *B. halodurans* PPKS-2. Gang et al. (2008) also revealed maximum keratinase activity by using casein, BSA, Feather meal and feather keratin with the organism *B. subtilis* KD-N2. When the relative activity of different substrates was compared it was observed that the substrates which were having more disulphide bonds could be easily hydrolysed than the substrates having less disulphide bonds, suggesting the solubility depends on the high percentage of disulphide bonds in the substrates (Moridshashi et al. 2020).

Different concentrations of the substrate keratin (1mM to10mM) were used to determine the Km and Vmax of purified keratinase. Km and Vmax values were determined by LB plot and were found to be 3mM-Km and 249µmol/mL/min Vmax respectively (Fig.7). Lower values of Km suggest high affinity towards the substrates indicating that the enzyme-substrate complex is tightly held before the substrate is converted to the product thus indicating that keratinase enzyme from *A. aneurinilyticus* has great affinity towards its substrate keratin. 1/S on the X-axis, 1/V on the Y-axis, a double reciprocal plot gave a straight line suggesting that our enzyme obeys the Michaelis Menton equation. Km being independent of enzyme concentration shows the characteristics of enzyme under defined temperature and pH condition. (Moridshashi et al. 2020).

Singh (2014) and Srinivasan (2008) also reported a low Km value of 0.01µg/ml/min and Vmax of 1176mg/ml with *B. subtillis* K-1. Dada (2020) also reported Km and Vmax kinetic constants of 25.60mM and 74.46U/ml respectively, with *B. licheniformis* K-51. Hydrolysis efficiency of 7mg/ml for Km and 384.6U/mg of Vmax was observed when casein was the substrate, while it was 7.2mg/ml and 103mg/ml respectively when the substrate was keratin azure with *Laeeyella sacchari* (Dada et al. 2020). Moridshashi et al. (2020) also reported a low Km value of 8.74mg/ml and Vmax of 59.04U/ml/min with feather meal substrate using *B. zhangzhouensis*. The above-said report correlates with ours (Dada et al. 2020).

## CONCLUSION

The findings of the present study showed that proteases of microbial origin are interesting, compared to plants or animal sources, as these enzymes from microbial origin possess all the features desired for biotechnological applications. Now, microorganisms are considered as efficient feather degraders. Consequently, keratin can be transformed by keratinolytic microorganisms such as *A. aneuinilyticus* with low molecular weight of (34KDa). Generally, the molecular weight of bacterial keratinases varied between species and combination of various peptidases is required for keratin degradation. Thus, keratinase with low mol. wt. can be

used in various biotechnological processes. Our studies of keratinase production from xerophytic endophytes will unravel the complex mechanism of keratinolysis and stability of the enzymes at high pH and temperatures which can be employed in various industries.

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**Biotechnological Communication**

# The Diversity and Biogeography of Haloalkaliphilic Bacterial Communities Producing Alkaliphilic Protease

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## ABSTRACT

Haloalkaliphilic bacteria are a specific group of bacteria known to us. The diversity of microorganisms is critical to the functioning of the ecosystem as there is the need to maintain ecological process such as decomposition of organic matter, nutrient cycling, soil aggregation and control of pathogens within the ecosystem. Microbial diversity as an indicator of the quality of agroecosystems has been widely debated, In the present study various saline soil samples collected from Bhavnagar and Uncha Kotda, Gujarat, India. The collected five samples were analysed for diversity study of soil sample for physicochemical analysis like pH, redox potential, conductivity, humidity, salinity and soil analysis for total nitrogen and organic carbon analysis also. Total 55 haloalkaliphilic bacterial morphotypes were isolated and screened for alkaline protease production on halophilic agar medium. Out of them 76.4% gram positive bacilli, 21.8% gram positive cocci and 0.0002% were gram negative short rods. From total 55 morphotypes 33% were different morphotypes, 8% were zone of casein producer, and 16% of them were pigment producing morphotypes. All the pigmented colony producer morphotypes showed growth of orange, yellow, red, light pink and light-yellow colonies on 15%, 20%, 25%, 30% NaCl containing medium. Dominantly bacilli were found in all five samples. Diversity indices for metabolic characterization studies like Shannon-weiner index (H'), Richness, Evenness, Cho-1, Simpson's index and Good's coverage were calculated based on the site wise obtained different morphotypes. Phenotypic characteristics were studied. The secondary screening and tertiary screening were done on the basis of REA and different NaCl concentration accordingly. Identification of all haloalkaliphilic protease producers were confirmed by 16S r-RNA identification.

**KEY WORDS:** CORRELATION ANALYSIS, DANDOGRAPH, K-MEANS CLUSTER ANALYSIS, SCATTER PLOT, STACKED BAR CHART.

## INTRODUCTION

Haloalkaliphilic organisms are essential for fundamental research and biotechnology perspectives (Ouelhadj et al. 2020). Extremophilic organisms have unique adaptation strategies that give them an integral role in the remediation of polluted sites. Haloalkaliphilic peptide degrading bacteria is one of those groups. Higher salt concentration is necessary for growth of haloalkaliphiles for optimum growth, which is making those morphotypes and their products more suitable for use in a variety of all pharmaceutical sectors (sMechri et al. 2019; Rathakrishnan and Gopalan 2022).

Haloalkaliphilic organisms have adapted physiological mechanisms to survive with high pH and salinity in these extreme environments. Haloalkaliphilic bacterial cells cope with the high salinity of the environment and compensate to prevent osmotic stress and water leakage. These organisms synthesise some osmoregulators of organic and inorganic compounds that avoid water loss. On the basis of intracellular accumulation of inorganic ions K+ and Cl-, halophilic archaea contains the salt in strategy which provide an osmotic balance (Mainka et al. 2021). Haloalkaliphiles are present in archaea, bacteria and eukarya all the three domains of life. Based on level of salt tolerance halophiles are classified as halotolerant, slight, moderate and extreme halophilic microorganisms. Halotolerant can grow, in saline environmenta but do not always required higher salt concentration for growth (Sysoev et al. 2021; Rathakrishnan and Gopalan 2022).

True halophiles are classified as slight (1-3% NaCl), moderate (3-15% NaCl) and extreme halophiles (15-30% NaCl) with comparision to sea water salinity approximately

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3.2% according to the salt concentration they require for the growth (Sysoev et al. 2021). Microbial biodiversity is the degree of variation of life forms within a biome, ecosystem and biome that measures the health of an ecosystem. Microbes maintain life over areas with extreme physicochemical conditions like hot springs, acidic springs, saline-alkaline lakes, hot and cold deserts, ocean beds that are harsh for life (Delgado-García et al. 2019). The enzymes isolated from saline bacteria have unique characteristics compared to non-halophilic bacterial enzymes (Rathakrishnan and Gopalan 2022).

Habitats of hypersaline environments are extreme with limited microbial diversity because of the combined effects of several environmental factors, including high salt concentrations, temperature, pH, low nutrient and oxygen availability(Ahmed and Mishra 2022). Biodiversity is a multidimensional property of a natural system (Santl-Temkiv et. al. 2022). Shannon and Simpson diversity indices include the measurement of variety and community heterogeneity. A Shannon index measuring the object of information theory is the content system order or disorder; frequently, it describes individual species' condition and uncertainty. The higher the uncertainty, the higher is the diversity that depends on two factors like the species richness and the evenness of species individual distribution. A large number of species can increase diversity (Davies et. al. 2022).

Diversity can uniformly increase with the uniform distribution of species among the different sites. If each individual belongs to another species, the diversity index is the largest, and if it belongs to the same species, its diversity index is the smallest. Diversity index is a quantitative measure that reflects how many different species in the community can simultaneously take into account the phylogenetic relations among the individuals distributed among those types (Davies et. al. 2022).

Simpson diversity index 'D' prospect that two randomly sampled individuals belong to different species. The more significant number of species in the community is the more uniform distribution of various individuals (Thumar and Singh 2009; Mahmood et al. 2022). The higher the index indicates a good diversity of the community. Simpson index is a scalar of α-diversity. The greater the Simpson index, the higher the diversity. Rare species play a minor role in this index, while common species play a more significant role. Simpson index is more weighted on dominant species than Shannon index (Mahmood et al. 2022). This study reports the cultivable bacteria diversity with particular reference to alkaline protease producers of saline soil from various samples collected from Bhavnagar and Uncha Kotda, Gujarat, India.

## MATERIAL AND METHODS

Five different saline soil samples were collected from various locations of the coastal area of Bhavnagar (21.7645 "N to 72.1519 "E) and Uncha Kotda (21.1274 "N to 71.9705 "E), Gujarat, India. Distance between the two points of the sample collection was about 30 to 50 m. Samples were collected in sterile zip lock pouches. Before analysing soil samples, all the soil samples were suspended in the distilled water (1:4 w/v) separately and allowed to settle the particles (Chaudhari 2013). Physico-chemical analysis like pH was measured using a glass-calomel combined electrode (HM digital pH meter, India) from the sample suspensions, and humidity was measured using a portable analyser (Infrared thermometer, India). The soil sample conductivity was analysed by a portable multi-meter analyser (Aquasol Power Max, India). Total nitrogen and organic carbon were measured accordingly by the Kjeldahl method and Walkley-Black chromic acid wet oxidation manual method (Vera-Gargallo and Ventosa 2018). Salinity was measured by a meter (Lutron salt meter PSA311, India).

For isolation all the five collected samples were separately added in sterile distilled water to prepare 10% w/v solutions, which were serially diluted up to 10-3 using sterile distilled water in triplicate. After serial dilutions, all the samples were plated on (i) haloalkaliphilic agar medium (Hi-media, India) supplemented with casein 5% (ii) Skimmed milk agar medium (iii) Alkaliphilic agar medium and Nutrient agar medium. All the media were supplemented with 10% NaCl and pH was adjusted to 10.5±0.5 using 1 N NaOH. After inoculation, all the plates were incubated at room temperature (34±4ºC) for 48-72 h. During the incubation period at regular intervals, colonies showing diverse visible morphological characteristics were picked up and transferred in the respective medium sequentially three to four times to get pure isolated culture.

All the pure morphotypes were preserved at 4ºC on the individual medium up to analysis (Vijayaraghavan et al. 2012; Vaishnav et al. 2014; Jothi et al. 2015; Maruthiah et al. 2015; Kim et al. 2017). In primary screening all the morphotypes were characterised based on the different morphotypes of colony characters, colonies with and without zone of casein hydrolysis, cell morphology, site-wise different types, total count and pigmented colonies. Moreover, the morphotypes were selected based on their growth in 10% NaCl and pH 10.5±0.5 containing alkaline media.

Alpha diversity marks concise the form of an ecological community concerning its richness and evenness as many contents affect the alpha diversity of a group summarising and comparing community structure by alpha diversity. In microbial ecology, analysing the alpha diversity of primer sequencing data is a common first approach to assess differences between environments. Based on the phenotypic characteristics of bacterial morphotypes, the Diversity indices, Shannon Weiner diversity index (H'), $R_{ichness}$ ($R_{margalef}$, $R_{menhinik}$) and Evenness ($E_{Pielou}$), Chao-1 were calculated using the standard formula (Oueriaghli et al. 2014).

Simpson's index (D) was also calculated for diversity, reciprocal and evenness. Good's Coverage was used for checking the percent of total species present in the samples (Martínez-Olivas et al. 2019; Sorokin et al. 2022). All the above-mentioned diversity indices were calculated using 'PAST' software (PAST 4.03, Paleontological Statistics).

Diversity indices were calculated on the basis of results of primary screening. Biochemical tests like the presence of endospore and capsule, production of catalase, oxidase, gelatinase, amylase and lipase, vancomycin test and 3% KOH test for Gram reaction were performed for all selected bacterial morphotypes (Chen et al. 2022). All the selected morphotypes were used to check relative enzyme activity (REA) and colony characters in secondary screening on 10% to 25% NaCl containing a haloalkaliphilic medium (Gaffney et. al. 2021). The morphotypes were selected for the tertiary screening based on the high REA results on different NaCl containing medium plates (Ariaeenejad et al. 2022).

The morphotypes from secondary screening were selected for final tertiary screening based on their growth in different NaCl containing broth and REA. In tertiary screening, protease production was checked in a production broth medium containing NaCl and alkaline pH. The salt concentrations studied was from 5% to 25%, while the medium pH tested were 9, 10, 11 at each NaCl concentration in the production medium. The composition of haloalkaliphilic protease production medium consist (g/L): yeast extract, 1.0; glucose, 6.0; malt extract, 1.0; $FeSO_4.7H_2O$, 0.1; $MgSO_4.7H_2O$, 0.5; $K_2HPO_4$, 0.5; peptone, 2; NaCl, 10.0; casein, 1.0 (Gupta et al. 2015). All the selected morphotypes were categorised as slight halophile or halotolerant (1-5% or 0.2-0.85 M NaCl), moderate halophiles (5-20 % or 0.85-3.4 M NaCl) and extreme halophiles (20-30 % or 3.4-5.1 M NaCl) (Bhatt and Singh 2017; Upadhyay et al. 2019; Asitok et al. 2022).

Species identification of morphotype were confirmed by 16S rRNA gene sequencing. The 16S rRNA gene sequences of selected all morphotypes were also submitted to the GenBank and sequence id accession number were obtained. All the obtained sequences were aligned with a multiple sequence alignment and phylogenetic tree of the morphotypes was constructed by the neighbour joining analysis using "Molecular Evolutionary Genetics Analysis Version 7.0 software (Saitou and Nei 1987; Kumar et al. 2016; Zuo et al. 2022).

## RESULTS AND DISCUSSION

Physico-chemical characters of collected soil samples: The results of the physicochemical analysis of all the samples are presented in Table 1. The pH of the samples collected from Bhavnagar ranged from 10.5 to 11.62, and samples from Uncha Kotda ranged from 11.12 to 11.73. The alkalinity of the samples was due to the presence of different salts present in the saline soil. The temperature at the time of sample collection was 34±5ºC. The redox potential of the samples collected from Bhavnagar ranged from 105 to 122 mV, and samples from Uncha Kotda ranged from 130 to 150 mV. The conductivity ranged from 15.69 to 36.9 mS and 13.08 to 18.7 mS for samples from Bhavnagar and Uncha Kotda, respectively.

**Table 1. Physico-chemical analysis of the samples**

| Parameters | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 |
|---|---|---|---|---|---|
| pH | 10.5 | 11.62 | 10.98 | 11.73 | 11.12 |
| Temperature (ºC) | 32.2 | 33 | 32 | 34.2 | 35 |
| Redox potential (mV) | 110 | 105 | 122 | 130 | 150 |
| Conductivity (mS) | 15.69 | 20.7 | 36.9 | 18.7 | 13.08 |
| Humidity (%) | 75 | 80 | 75 | 56 | 49 |
| Total nitrogen (mg/kg) | 0.23 | 0.26 | 0.18 | 0.28 | 0.32 |
| Organic carbon (%) | 0.73 | 0.79 | 0.80 | 0.99 | 0.96 |
| Salinity (mg/L) | 4890 | 20800 | 26500 | 4980 | 3640 |
| |  |  |  |  |  |

Total nitrogen and organic carbon ranged from 0.18 to 0.32 mg/kg and 0.73 to 0.96% respectively. While salinity of the samples from Bhavnagar ranged from 4890 to 26800 mg/L, and samples from Uncha Kotda ranged from 3640 to 4980 mg/L. Salinity and conductivity have a linear relationship. Results here mentioned in table 1. As salinity increases, the conductivity of the sample also increases (Rusydi, 2018). Samples (1, 2 and 3) from Bhavnagar showed more redox potential, salinity, and conductivity than samples (4 and 5) from Uncha Kotda. The results measured here are of one specific day of sample collection, and hence the variation was due to the different sample collection sites. The collected saline soil samples were diverse in physicochemical characters and therefore showed variation in the results from site to site. The results indicated the presence of dissolved solids, salts and impurities in the form of hydroxides, carbonates and bicarbonates with sodium chloride imparting a slightly alkaline nature to the habitat (Dave and Desai, 2006). Moreover, Bhavnagar and Uncha Kotda are situated 84 km away from each other.

**Isolation of haloalkaliphilic morphotypes:** The haloalkaliphilic morphotypes were isolated from saline soil of Bhavnagar and Uncha Kotda, Gujarat, India. Total 55 morphologically different types (morphotypes) of bacteria were isolated on a 10% NaCl containing medium. Of the

obtained bacterial morphotypes, 9.18% were halotolerant, 9.6% were moderate halophiles, and 11% were extreme halophiles. Table 2 shows the site-wise total count of obtained bacterial morphotypes.

**Table 2. Site-wise total bacterial count on various media**

| Medium | Total viable count (CFU/ g) | | | | |
|---|---|---|---|---|---|
| | Sample number 1 | 2 | 3 | 4 | 5 |
| Haloalkaliphilic medium (Jothi et al. 2015) | $5.65\times10^4$ | $8.01\times10^4$ | $9.06\times10^4$ | $2.36\times10^4$ | $2.89\times10^4$ |
| Alkaliphilic medium (Vijayaraghavan et al. 2012) | $0.68\times10^3$ | $0.044\times10^3$ | $0.88\times10^2$ | $0.03\times10^2$ | $0.5\times10^2$ |
| Skimmed milk agar medium (Vaishnav et al. 2014) | $0.12\times10^1$ | $0.03\times10^1$ | $0.05\times10^1$ | $0.4\times10^2$ | $0.02\times10^2$ |
| Nutrient agar medium | $0.7\times10^1$ | $0.5\times10^1$ | $0.3\times10^1$ | $0.8\times10^1$ | $0.01\times10^1$ |

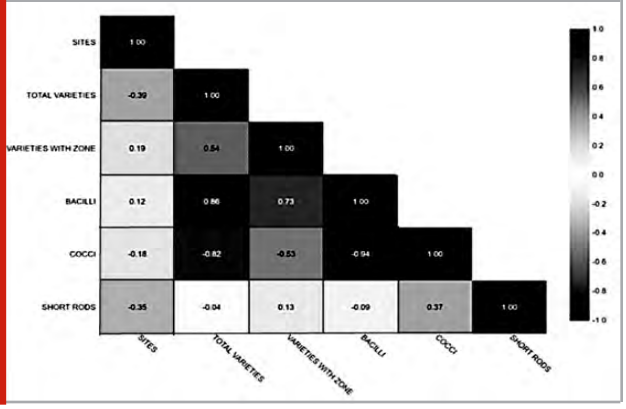
Saline soil samples collected from Bhavnagar showed more morphotypes as compared to Uncha Kotda. The majority of the morphotypes were found present in each sample. Table 2 shown the site-wise bacterial count results obtained on the studied different media, and the haloalkaliphilic medium containing 10% NaCl and 5% casein was the most suitable one. Site 3 showed the highest 9.06×104 CFU/g. In contrast, skimmed milk agar and nutrient agar medium resulted in the lowest bacterial count. Haloalkaliphilic agar medium was selected as an appropriate medium for the isolation and further screening of the bacteria from the saline soil.

**Primary screening:** Colony and cellular characters were recorded in terms of all the colony characters like size, shape, margin, elevation, texture, opacity and pigmentation as mentioned in Table 3, with particular prominence on pigment colour and casein degradation zone.

Among 55 morphotypes, cellular characteristics showed the dominance of 39 gram-positive bacilli, and the remaining were 15 gram-positive cocci and one gram-negative short rod. The obtained morphotypes showed growth on 10, 15 and 20% NaCl concentrations. All the samples showed the presence of a wide variety of haloalkaliphilic protease producing bacteria. A total of 40 morphotypes showed a zone of casein hydrolysis that were screened out after primary screening. Morphologically different morphotypes with a zone of casein hydrolysis were used for secondary screening. Site-3 gave the highest CFU/g (Table 2) of the collected soil sample. Site-1 gave the highest number of bacterial morphotypes. It was also observed that sample 1 showed the maximum bacterial diversity at alkaline pH and higher NaCl concentration. The colony characters of 55 morphotypes are mentioned in Table 3. Many morphotypes were common to all sites. Out of these 55 morphotypes, 41 were gram-positive bacilli comprising 76.4%, 13 gram-positive cocci (21.8%), and only one isolate (0.0002%) gram-negative short rod (Qu et al. 2022).

Site-wise total morphotypes, morphotypes with a zone of casein hydrolysis and their morphology are mentioned in Figure 1 of correlation analysis (Minitab software). Figure 1 depicts that the correlation coefficient ranges from -0.94 to 1.0. We found maximum morphotypes of bacilli as compared to cocci and short rods, respectively. Bacilli to cocci correlation observed were -0.94 and -0.09 in the case of bacilli to short rods. Total morphotypes found were between -0.82 to 1.0, bacilli with 0.80, cocci with -0.82 and short rod with -0.04. This analysis suggests the diversity of species similarity of all sites. From total morphotypes, casein hydrolysing zone forming morphotypes were in the range of 0.19 to 1.0. Halophilic morphotypes are pleomorphic as they can survive in high NaCl concentrations (Patel et al. 2006; Yadav and Patil 2020).

**Figure 1: Correlation analysis of 55 morphotypes based on the zone of casein hydrolysis and their morphology**



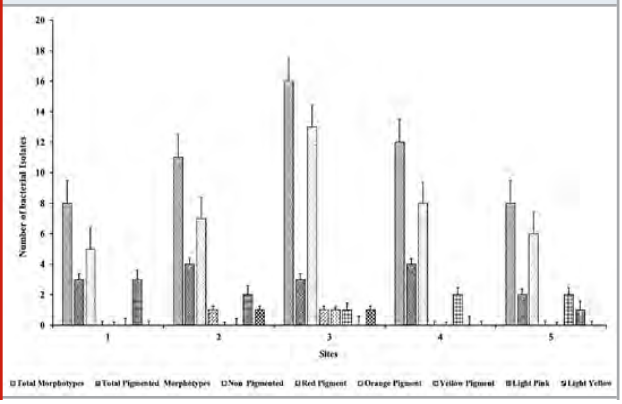

Halobacteriales needs high salt concentration for their structural steadiness. The non-coccoid forms of bacterial structure lie under 10% NaCl concentration (Dave and Desai 2006). Here majority of morphotypes were gram-positive *bacillus* (Qu et al. 2022). This statistical (Minitab 20) technique was used to determine the dependence between two or more variables. Here this correlation design shown a positive correlation ship between all variables. It offers a strong dependency relation of variables pairs. The extent to which two variables vary together were also determined apart from the relationship of strength and direction.

**Table 3. Colony characters observed on casein agar plates (pH 10.5) from samples**

| | Colony characters | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Morphotypes | Size | Shape | Margin | Elevation | Consist | Opacity | Texture | Pigment |
| 1* | Small | Round | Entire | Raised | Moist | Translucent | Smooth | Yellow |
| 2* | Big | Round | Wavy | Flat | Dry | Translucent | Rough | White |
| 3 | Small | Round | Entire | Raised | Mucoid | Translucent | Smooth | Nil |
| 4 | Big | Round | Entire | Raised | Mucoid | Translucent | Smooth | Yellow |
| 5 | Small | Round | Irregular | Flat | Mucoid | Translucent | Rough | Nil |
| 6 | Small | Round | Irregular | Convex | Mucoid | Opaque | Smooth | Nil |
| 7* | Small | Irregular | Entire | Flat | Dry | Translucent | Dry | Nil |
| 8 | Big | Irregular | Irregular | Flat | Mucoid | Translucent | Smooth | Light pink |
| 9* | Small | Pinpoint | Entire | Flat | Dry | Translucent | Dry | Yellow |
| 10* | Big | Irregular | Irregular | Flat | Dry | Translucent | Rough | Yellow |
| 11* | Small | Round | Irregular | Raised | Moist | Opaque | Smooth | Yellow |
| 12* | Big | Irregular | Irregular | Flat | Dry | Translucent | Swarming | Nil |
| 13 | Big | Big | Convex | Raised | Moist | Opaque | Smooth | Light yellow |
| 14* | Small | Round | Entire | Flat | Dry | Translucent | Rough | Nil |
| 15* | Small | Round | Entire | Raised | Moist | Opaque | Smooth | Orange |
| 16* | Small | Round | Entire | Flat | Dry | Translucent | Swarming | Nil |
| 17* | Big | Oval | Entire | Raised | Mucoid | Opaque | Smooth | Orange |
| 18* | Small | Round | Entire | Flat | Dry | Translucent | Rough | Pink |
| 19* | Small | Round | Entire | Raised | Moist | Opaque | Smooth | Red |
| 20* | Small | Round | Entire | Flat | Dry | Translucent | Swarming | Nil |

Note- '*' for colonies with a zone of casein hydrolysis.

**Figure 2: Site-wise morphotypes of pigmented bacteria compared with the total number of isolate and non-pigmented morphotypes.**




**Site wise study of pigmented morphotypes:** Out of 55 morphotypes, 33% were different morphotypes, and from all of them, 8% of morphotypes showed a zone of casein hydrolysis, and 16% of the morphotypes produced a variety of pigments. Among the 55 morphotypes and 40 different significant casein hydrolysis zone-producing morphotypes that could grow at 10%, NaCl concentrations were selected further. Colonies of the selected 55 morphotypes showed various pigment formations like orange, red, pink, light pink, yellow on Haloalkaliphilic agar medium plates. Figure 2 represents the comparison of total bacterial morphotypes with pigmented and non-pigmented bacterial morphotypes. Sample from site 3 showed the highest number of total bacterial morphotypes with pigmented colonies. Site 2 and site 4 equally showed the highest number of pigmented colonies than the other sites (Qu et al. 2022).

Light pink-coloured bacterial morphotypes were dominating among all the morphotypes. All the pigmented bacterial morphotypes were gram-positive bacilli or cocci. Generally dominant red and orange pigmented colonies presented at 25% NaCl, pink at 20%, yellow at 15%, whereas colourless colonies dominated at 10% NaCl concentration. The pigment intensity and pigment producer morphotypes increased with NaCl concentration in the medium (Dave and Desai 2006; Purohit et al. 2016; Qu et al. 2022). Here we can conclude that in all the collected saline soil samples, greater diversity in the bacterial colony observed on haloalkaliphilic agar, indicating that obtained morphotypes could be halophilic (Hegazy et al. 2020).

**Study of diversity indices:** The results of various diversity indices are shown in Figure 3. Shannon Weiner index (H') ranged from 2.9 to 4.39. Shannon's diversity index for bacterial community reported from 2.97 to 5.48 for microbial diversity (Maron et al. 2018). Shannon's index

was also reported 1.47 to 2.04 for Halomonas community from saline soil of Rambla Salada (Oueriaghli et al. 2014). Samples 1 and 5 had a lower value of Shannon Index above 2, whereas samples 2, 3 and 5 had 3.24, 4.39 and 3.89 accordingly. Shannon's evenness index reported as 2.3 (Sharma et al. 2021) whereas, our results showed evenness that ranged from 1.26 to 2.62. Site 3 offered the highest evenness of 2.62. Simpson's evenness for haloalkaliphilic archaea reported from 0.40 to 0.78 from marine samples (Martínez-Olivas et al. 2019; Hegazy et al. 2020).

**Figure 3: Site-wise diversity indices calculated based on species observations**

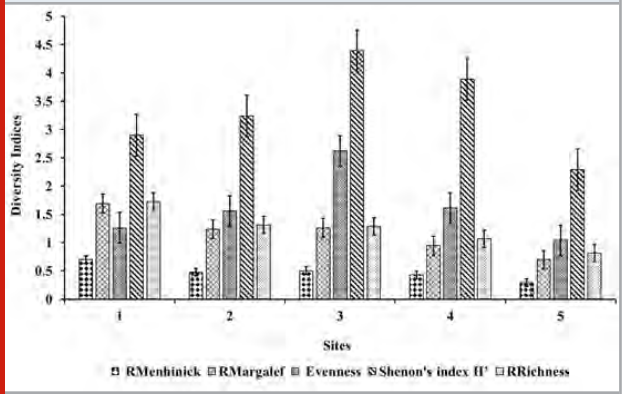
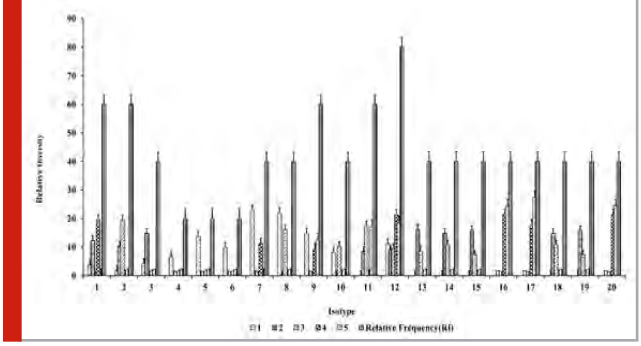



**Figure 4: Study of relative frequency and relative density of totally different morphotypes**




Richness ranged from 0.8 to 1.73, $R_{Margalef}$ ranged from 0.7 to 1.69, and $R_{Menhinick}$ ranged from 0.291 to 0.7. Bacterial diversity richness reported from 313 to 1004 (Maron et al. 2018). For lignite mine bacteria, $R_{Margalef}$ reported from 0.0 to 15.88, and $R_{Menhinick}$ reported from 0.87 to 5.89 (Patel et al. 2009; Maron et al. 2018). In our results, site 1 showed the highest species richness, whereas site 5 showed the lowest species richness than other sample collected sites. $R_{Margalef}$ and $R_{Menhinick}$ of 2.3 and 1.7 for haloalkaliphilic actinobacteria had been reported (Sharma et al. 2021). Relative frequency and relative density depicted in Figure 3 concerning obtained total morphotypes from all 5 sites (Sharma et al. 2021).

Variety no. 12 (Table 3) showed the highest relative frequency among all sites in the study. Site 1 gave relative density ranged from 3.8 to 23.07, whereas sites 2, 3, 4 and 5 showed relative density ranged from 8.42 to 16.34, 7.3 to 19.57, 9.25 to 21.29 12.59 to 24.44, respectively. Isotype 12 showed the highest relative frequency of presence to all the respective 5 sites. Colony types 1-10 and 12 were observed in sample 1 from site-1. Colony types 1-3 and 11-15 were present in sample 2 from site-2. Colony types 2, 8, 10-15 were observed in sample no. 3 from site-3. Colony types 12, 16-20 were present in sample no. 4 from site-4. Colony types 12, 16, 18 and 20 were present in sample no. 5 from site-5. Colony numbers 1-4 and 11 showed similar colony morphology visually that were present in samples 1 and 2 (Sharma et al. 2021; Li et al. 2022). Colony numbers 2 and 10-15 were morphologically similar colonies in samples 2 and 3. Colony no. 12, 16-20 were present in samples 4 and 5. Morphotype 12 was dominantly present in all the sites. Figure 5 scatter plot showen the site-wise presence of different morphotypes based on colony morphology.

**Figure 5: Site wise morphotypes (based on colony characters)**

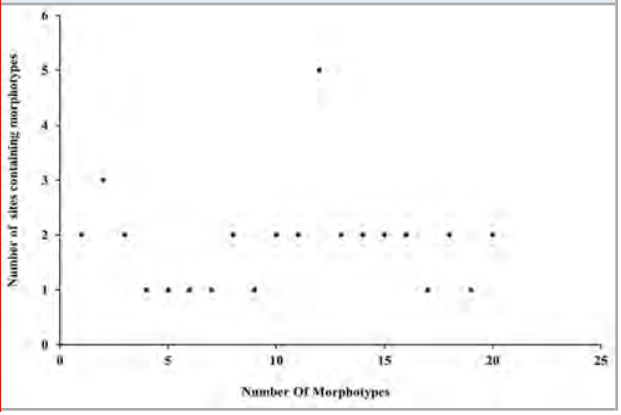
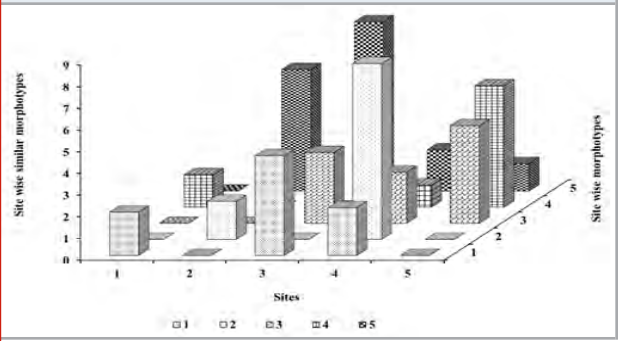



**Figure 6: Site wise similarity index**




The similarity index (Figure 6) could be explained based on the ecosystem of the respective site. Site 1 showed the highest richness and diversity indices, while site 3 showed the least richness and diversity of bacterial morphotypes. All the other indices calculated were in support of these observations. Chao1 abundance-based species richness estimator for missing morphotypes was 1.3 from species morphotypes. From the study of all the diversity indices, it has been determined that sample number 1 had overall low density, whereas sample number 3 showed high density, evenness and richness.

Simpson's index (D) is shown in Figure 7. It was demonstrated the numerical proportion of similarity probability between various randomly selected species. It ranges from 0 to 1 with representing infinite diversity and no diversity, respectively. The higher the value of Simpson's diversity index greater the ecological diversity. The ecological values of Simpson's index site-wise were 0.4, 0.98, 0.97,0.98 and 0.98 accordingly. The Simpson's index of diversity 0.78 shown rich haloalkaliphilic bacterial diversity. Simpson's index has been reported for haloalkaliphilic archaeal microbial diversity from 0.70 to 0.97 and evenness from 0.07 to 0.48 (Martínez-Olivas et al. 2019). Simpson's reciprocal index site-wise from site 1 to 5 were 25.08, 59.7, 55.4, 60.24 and 40.81 accordingly. For haloalkaliphilic bacterial archaea. It has been reported from 27.08 to 61.27 (Martínez-Olivas et al. 2019; Sharma et al. 2021).

**Figure 7: Simpson's diversity index-based comparison between the selected sites**

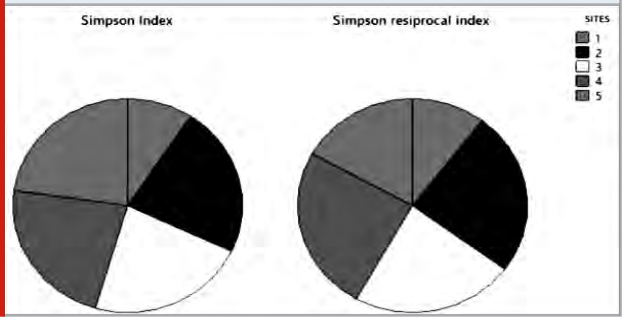
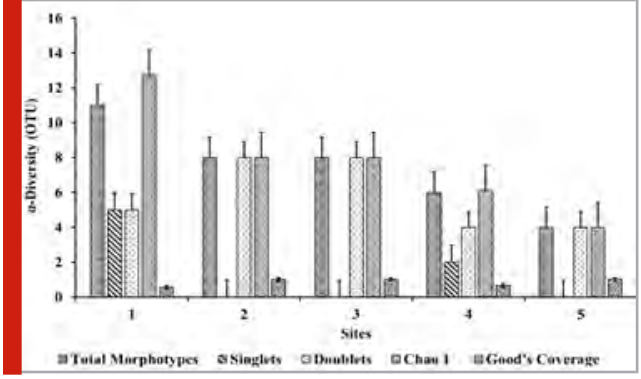



**Figure 8: Site-wise Chao 1 analysis**

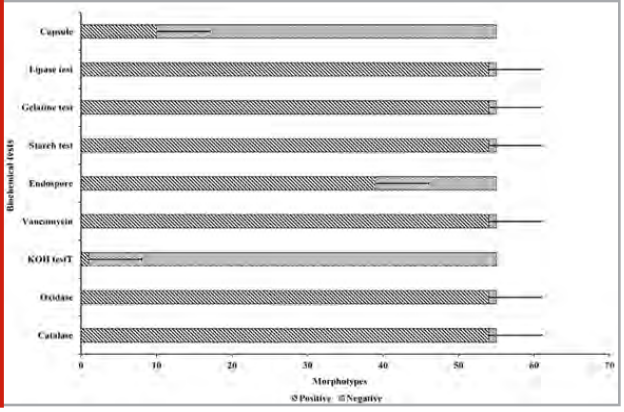



From the Shannon's and Simpson's indices studies, it was observed that most diverse bacterial morphotypes were able to grow at different salt concentrations with a variety of pigment production could be the reason for its high diversity value (Li et al. 2022). There was a need to evaluate how well a sample reflects the true diversity of a specific niche. The diversity of a particular niche always synonymouses with species richness and relative abundance in time and space. Accurate assessment of species richness is instrumental for each biological community. With other indices, Chao1 was a nonparametric method for estimating the number of species in a community (Farheen et. al. 2022). Chao 1 was based on the concept that rare species infer most information about the missing species as the Chao 1 richness estimator gives more weight to the low abundance of the singletons and doubletons were used for estimation number of missing species (Oosterkamp et al. 2019; Sikorski et al. 2022).

Site-wise missing morphotypes (Figure 8) were ranged from 1.2 to 10.5. Site 1 was shown the highest value of Chao-1 analysis 12.7, whereas site 5 was shown a lower value of Chao 1 analysis 4. Site 1 revealed maximum variables and missing morphotypes of bacterial morphotypes. Oosterkamp et al. (2019) reported the Chao-1 index was from 410 to 1091 range for microbial community diversity. Whereas, for haloalkaliphilic archaeal diversity Chao-1 index of 70 to 349.14 was given by Martínez-Olivas et al. (2019). Where Zhang et al. (2018) reported Chao-1 index from 74.33 to 799.0 for microbial diversity.

Good's coverage (Coverage= 1-(singlets/total morphotypes)) of all the sites 1 to 5 was calculated as, 0.37%, 0.32%, 0.67% and 0.63% respectively. It estimates the percentage of total species present in the sample. It was an alpha diversity metric. Good's coverage was reported for bacterial diversity in the range of 0.50% to 0.82% and halophilic archaea 0.55% to 0.94% (Martínez-Olivas et al. 2019). Moreover, Zhang et al. (2018) reported Good's Coverage value ranged from 0.38% to 2.47% for microbial diversity.

**Figure 9: Cluster bar chart for the biochemical test of 55 morphotypes**




**Phenotypic characterisation of organisms:** Figure 9 represents the cluster bar chart for all 9 biochemical tests. This analysis showed a pictorial representation analysis of biochemical tests of 55 bacterial morphotypes obtained on the haloalkaliphilic agar medium. Clustered bar chart allows the direct comparison of multiple data series per category, which shown change over time. Each sector denotes a compatible part of the whole. The figure represented the categorical data of catalase, oxidase, KOH, vancomycin, endospore, capsule, gelatine degradation, lipase production, and starch hydrolysis tests. Total 54 morphotypes showed catalase, oxidase, KOH, vancomycin, gelatine, lipase and starch hydrolysis test positive. At the same time, one isolate showed these mentioned tests negative. Sixteen morphotypes showed the formation of endospore, and 39 gave the test negative. Ten morphotypes showed the presence of a capsule, and 45 morphotypes were non-capsule former. It offers the inner products between the

observations and variables. One bacterial isolate showed a difference in most biochemical tests in the plot that correlates to the Chol-1 index whereas, the rest of the bacterial morphotypes showed similarity. It could be due to its various metabolic properties (Sikorski et al. 2022).

**Secondary screening based on REA:** After the primary screening, all the obtained 40 morphotypes spreaded on the haloalkaliphilic agar medium of pH 10.5±0.5 and supplemented with 5% skimmed milk containing different NaCl concentrations of 10% to 25% (Figure 10). Site 3 was found rich in a number of bacterial morphotypes shown zone of casein hydrolysis at 15%, 20% and 25% NaCl concentration. Therefore, 20 morphologically distinct morphotypes were selected for the salt tolerance test from 20 % NaCl and pH 10.5±0.5 containing haloalkaliphilic agar medium with higher REA activity. The relative enzyme activity (REA) of the selected 40 morphotypes shown in Figure 11. REA values of 21 to 40 mm giving 20 morphotypes were further selected for the haloalkaliphilic protease production test.

**Figure 12: REA of selected morphotypes**

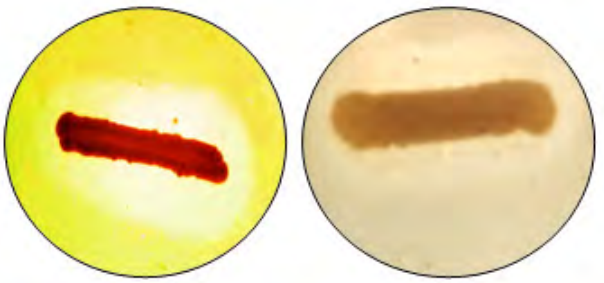
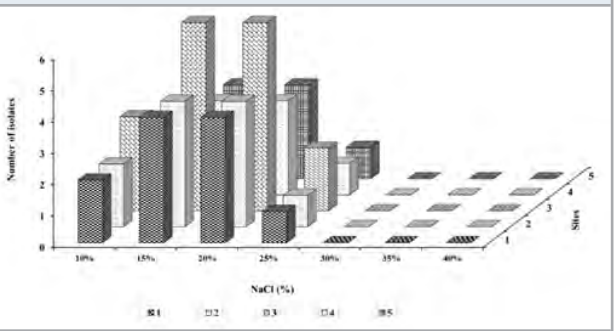


**Figure 10: Secondary screening based on REA**



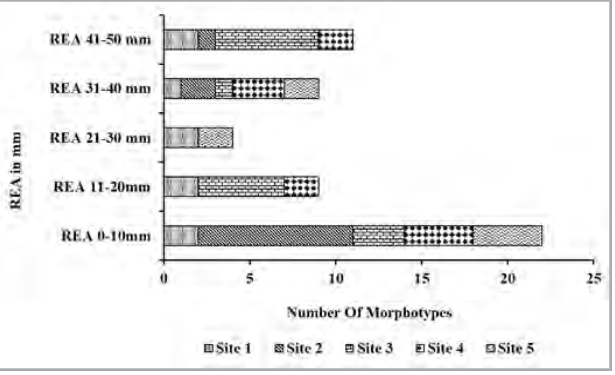

Based on REA, the morphotypes can be categorised into three groups. (REA= Zone diameter of casein hydrolysis/ Colony diameter in mm). REA>5mm is magnificent, REA >2 to 5 mm is satisfactory and REA<2 is the deficient producer of protease (Jadhav et al. 2016). Here, the zone of casein hydrolysis on haloalkaliphilic agar medium shown the presence of protease producing bacteria. Some strains showed clear hydrolysis zones around the bacterial colonies on the haloalkaliphilic agar medium with 10% NaCl, which indicates a dominant amount of protease (Cui et al. 2015; Sikorski et al. 2022).

The salt and pH requirements vary among the morphotypes even obtained from the same site indicating extensive diversity. From Figures 10 and 11, it can be concluded that a few of the morphotypes from 2B, 4I, 3K, 4D, 3E, 5F, 1D, 4B, 2C, 1G 1C, 3I, 1E, 3C, 5H, 2D, 5B, 3A, 3H, 4A might be the good haloalkaliphilic protease producers. Site 3 from Bhavnagar showed the highest six morphotypes among the selected best at 15-20% NaCl and REA 31-50 mm. In contrast, site 5 from Uncha Kotda showed fewer morphotypes compared to other sites at extreme conditions. The number of haloalkaliphilic bacterial morphotypes and diversity can be lower at the same site because of an increase in the extremity of pH and salt during the enrichment process of isolation (Purohit et al. 2014; Bhatt et al. 2018). Figure 12 shown a clear zone of casein hydrolysis of selected morphotypes (Sikorski et al. 2022).

**Tertiary screening for haloalkaliphilic protease production with different pH and NaCl concentrations:** Significant haloalkaliphilic protease producing bacteria were selected based on REA. Figure 13(a, b) shown the fermentative production of haloalkaliphilic protease.

Selected 7 morphotypes denoted as 1D, 2B, 3E, 3K, 4D, 4I and 5F were checked for haloalkaliphilic protease production at different pH (9, 10 and pH 11), and NaCl concentrations (10%, 15%, 20% and 25%). At least one isolate from each site was selected. Further, the isolate that gave the best result at pH 11 and 20% NaCl concentration within 24 h will be used to optimise the enzyme production. The tertiary screening results based on pH 11 and 20% NaCl concentration, isolate 2B, 3E, 4I, 5F and 3K were selected for further experiments.

**Figure 11: REA of morphotypes (Stacked bar) on the basis of zone of casein hydrolysis**




**Phylogenetic analysis of the morphotypes:** The biochemical tests determined the metabolic activity of the bacterial morphotypes. 16S rRNA gene sequencing of selected morphotypes were used for the identification of bacteria and phylogenic analysis as the "optimum mark". It was reported that molecular based identification of morphotypes by 16s rRNA gene sequencing helps at the genus level identification (Patel et al. 2019). On the basis of 16S rRNA partial gene analysis selected isolates represented 24 different genera and species of that genera. By this way it was showed morphological and metabolical

diversity. Pareto plot analysis of the morphotypes showed that most of the morphotypes belonged to the genus *bacillus* species and identified as *Bacillus halodurans, Bacillus megaterium, Bacillus subtilis, Bacillus oxytoca, Micrococcus, Planococcus, Halomonas, Pedicoccus, Oceanobacillus* spp., *Exiguobacterium, Bacillus cereus, Enterobaceter aerogenes, Bacillus thermophiles, Lactobacillus mali, Bacillus licheniformis, Penibacillus, Bacillus oryzaecoeticis, Lysinibacillus, Bacillus pumilis, Bacillus coagulans, Halobacterium salinarum, Bacillus halodurans, Bacillus firmus, Bacillus awamori, Bacillus halodurans, Bacillus qingdaonensis. Bacillus halodurans* was present frequently in all the sample collection sites (Sikorski et al. 2022).

**Figure 12: Tertiary screening of different pH and NaCl concentration (a) pH (b) NaCl**

Figure 13(a) Figure 13 (b)

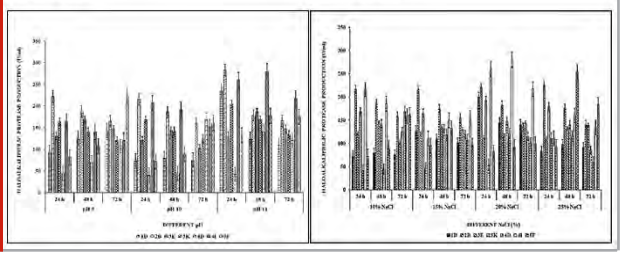
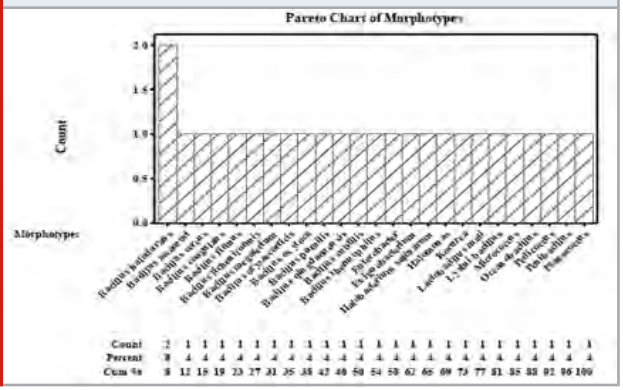


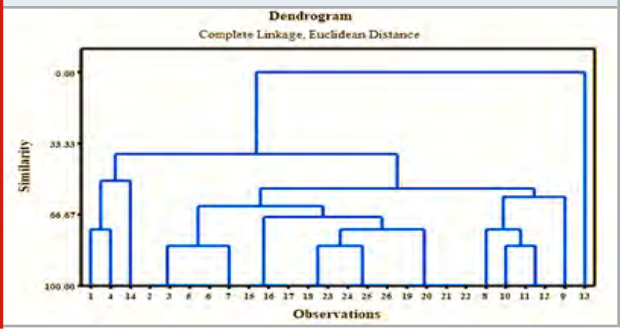
**Figure 13 Pareto plot for bacterial phylogenetic analysis**




All the morphotypes were gram positive except one gram negative morphotype Enterobacter. Rest of the other morphotypes were present distinctively at all the sites. Based on 16S r-RNA gene analysis, evolutionary relationship between cultivable morphotypes isolated from different locations of Bhavnagar city and Uncha kotda Gujarat, India.

Tree diagram of taxonomic relationship for site wise morphotypes with distance and similarity shown in the figure 15. The hierarchial clustering algoritham shown similar objects into cluster groups.The end point was a set of clusters which was distinct from each other clusters and objects within each cluster are broadly similar to each other. Here, site 1, 2, 3, 4 and 1, 4 and 5 were showing diversity similarity which matches to the similarity results of the Simpson's diversity index results mentioned above.The evolutionary distance of morphotypes is depicted by the length of the horizontal line (Daly et al. 2018).The branch point similar with the horizontal line was the divergence point of two microbial species (Mulango et al. 2020). On the basis of this diversity studies it has been concluded that the hypersaline soil sample containing all the sites were augmented with a vast number of morphotypes showing divergent metabolic potential (Sikorski et al. 2022).

**Figure 15: Cluster dandogram showing relationship amongst all morphotypes Hierarchical Cluster analysis of taxonomic identification**




## CONCLUSION

The study of culture-dependent biodiversity from Bhavanagr and Uncha Kotda from Gujarat coast showed species richness and evenness. Site 3 from Bhavnagar was offering the highest variation in the diversity study. Moderately halophilic bacterial morphotypes were selected for further analysis. Haloalkaliphilic bacteria are capable of showing function under higher alkaline pH and salt conditions. They showed diversity based on their colony correlated cultural and morphological features, gram reaction, biochemical properties, secretion production of enzymes and metabolites. Diversity, physiology and metabolic studies of haloalkaliphilic bacteria help understand biopower and surveillance of these organisms under extreme conditions. Here, we can conclude that in all the collected samples, we observed a greater number of cultivable morphotypes with different pigments on haloalkaliphilic agar that indicate isolated morphotypes to be halophilic bacteria. Further halophilic aspects of morphotypes will be confirmed by SDS-PAGE analysis by correlation of molecular weight of the isolated morphotypes with standard molecular weight of haloalkaliphiles. All selected five morphotypes that indicated a potential for high production of haloalkaliphilic protease would be used for scale-up of process application.

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# Molecular Genetic of Hemochromatosis Disease using Bioinformatics Tools

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## ABSTRACT

Hemochromatosis is caused by p.Cys282Tyr mutations in HFE. This study's objective was to find causal or disease-related variations in people with erythrocytosis of unknown origin who came from a family with clear blood markers and other indicators of congenital erythrocytosis. This research aims to create a new hemochromatosis risk prediction prototype and evaluate psychographic, clinical, and genomic data to improve predictive model performance. In this review, a conditional characterization of primary iron overload, secondary iron overload, and hemochromatosis medical history is established, as well as an analysis of the drug molecules used to treat hemochromatosis. This paper provides Hemochromatosis Gene brand and its operation.

**KEY WORDS:** HAEMOCHROMATOSIS, HFE GENE, IRON-OVERLOAD, PHLEBOTOMY, THERAPY.

## INTRODUCTION

Hemochromatosis is a metabolic disorder in which the body dissolves excessive amounts of iron from food. Increased iron levels in the blood poison the tissues of the liver. Pancreas, heart, pituitary gland, joints, and skin. Hemoglobin is found in millions of copies in red blood cells. Which binds to oxygen and converts into tiny oxygen transporters that allow oxygen to reach all of our body's tissues (Whitlock et al. 2006). These hemoglobin proteins are made up of four Hemi-molecular, each of which contains iron in the midsection (McLaren et al. 2003). We commonly waste about 1 mg of iron per day, some through sweat, some through shed skin cells, and some through shedding gastrointestinal tract cells. Most of us consume 10 to 20 mg of iron a day throughout our diets and accumulate just about 10% of it (Adams et al. 2005). Hemochromatosis patients accumulate an abnormally high amount of iron. You can take up to 4 mg per day, despite the fact that you only need about 1 mg to compensate for your losses (Kirk et al. 2009; Ong et al. 2017).

A net gain of 3mg per day equates to about 1g of excess iron in our bodies per year, leading to more than 20mg by age 40. The liver stores the most iron, but it's also found in the pancreas, heart, joints, skin, and pituitary gland. Unfortunately, all of this extra iron causes significant harm because iron in the body is quite useful at producing free radicals via the Fenton reaction (European Association for the Study of the Liver 2010). This reaction occurs when iron2+ molecules are oxidised by $H_2O_2$, resulting in iron3+. Iron3+ can then be decreased back to iron2+ by $H_2O_2$, resulting in a peroxide radical, accomplishing an infinite loop of free radicals (Bardou-Jacquet et al. 2015). As a result, all of these iron deposits produce free radicals in the cells of different organs over time, which can result in cell death and tissue fibrosis (Ong et al. 2017).

**Figure 1a**


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**Figure 1b**

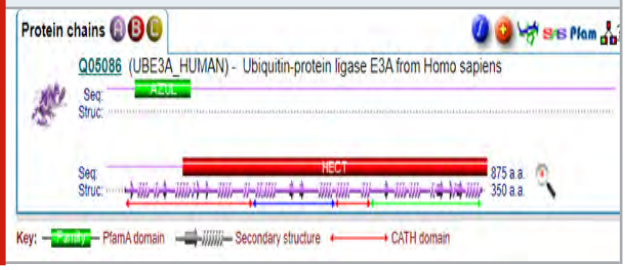
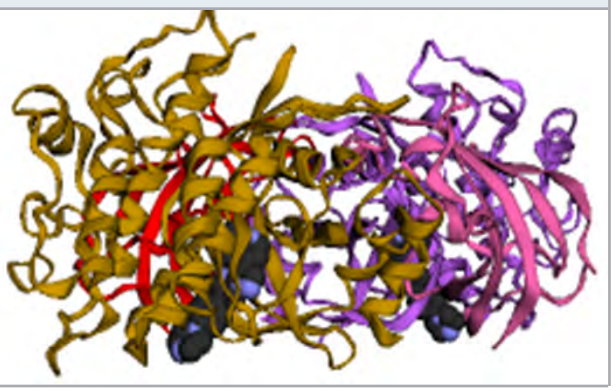



**Figure 1c**



**Types of Hemochromatosis:** Hemochromatosis is two type primary and secondary. PRIMARY HEMOCHROMATOSIS: Hereditary hemochromatosis is another name for primary hemochromatosis. Mutations in the HFE gene, which is found on chromosome 6, cause it. This is beneficial for controlling the amount of iron we absorb from our food. The C282Y mutation or the H63D mutation is present in people with this autosomal recessive disorder. This mutation affects enterocytes, which are absorptive cells in the small intestine that absorb a variety of substances as well as iron. The iron is absorbed when it is required. They basically control how much iron enters the bloodstream from the intestine. Because these enterocytes are no longer as effective at regulating iron, the majority of the iron in your diet simply passes through the bloodstream, overloading the blood (Olynyk et al. 1999; Milman et al. 2003; Ong et al. 2017).

**Secondary Hemochromatosis**: Secondary hemochromatosis occurs when hemochromatosis is caused by something other than a genetic mutation. Frequent blood transfusions are an example of secondary hemochromatosis. When you receive new blood via transfusion, those red blood cells die after about 120 days. Because the iron in blood is used again, each new bag effectively brings a package of iron to your body. Blood products result in a high level of iron in the blood (Widdowson and McCance 1937; Ong et al. 2017).

**Figure 1:**

| Gene type | Gene symbol | OMIM | underlying condition | Hepcidin intensity |
|---|---|---|---|---|
| type 1 | HFE | 613985 | Primary iron overload | Minimal |
| type 2A | HFE2 | 602390 | Primary iron overload | Very low |
| type 2B | HAMP | 613313 | Primary iron overload | Minimal to average |
| type 3 | TFR2 | 604250 | Primary iron overload | Minimal |
| type 4A | FPN1 | 606069 | Primary iron overload | Minimal to average |
| type 4B | FPN1 | 606069 | Primary iron overload | Strong |
| β-thalassemia | HBB | 613985 | Hemoglobinopathy | Minimal to average |
| Sickle cell anemia | HBB | 603903 | Hemoglobinopathy | Minimal to average |
| X-linked sideroblastic anemia | ALAS2 | 300751 | Hemoglobinopathy | Minimal |
| Pyruvate kinase deficiency | PKLR | 266200 | Hemolytic anemia | Minimal |
| Hereditary spherocytosis | Heterogenous | 182900 | Hemolytic anemia | Minimal |
| Friedreich ataxia | FXN | 229300 | Mitochondrial iron overload | Unknown |
| Hereditary atransferrinemia | TF | 209300 | Plasma protein deficiency | Minimal |
| Hereditary aceruloplasminemia | CP | 604290 | Plasma protein deficiency | Minimal |

HFE stands for Homeostatic Iron Regulator; HAMP stands for Hepcidin Antimicrobial Peptide; TFR2 stands for Transferrin Receptor 2; FPN1 stands for Ferroportin-1; and HBB stands for Hemoglobin Subunit Beta. ALAS2 =5'-Aminolevulinate Synthase 2; PKLR =Pyruvate Kinase, Liver and Red Blood Cell: FXN stands for Frataxin, TF stands for Transferrin, and CP stands for Ceruloplasmin.

**Characterstics of the Disease:**

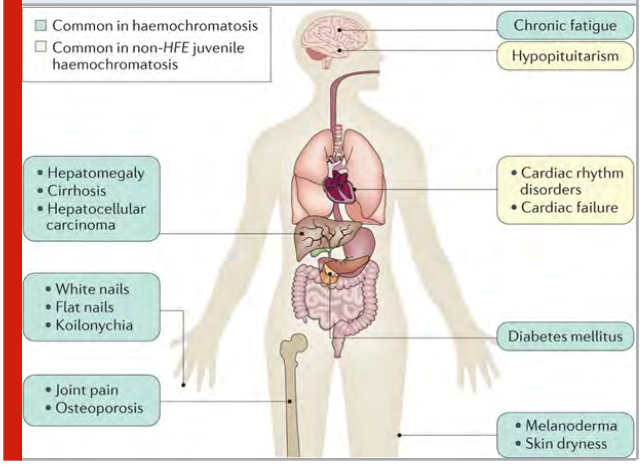
The physical feature of Hemochromatosis include:

- Unexplained weight loss
- Pain in joints, knuckles
- Loss of sex drive
- Loss of body hair
- Foggy memory
- Heart flutter
- Feeling tired
- Skin that has a bronze or grey colour

**Table 2. Shows Gene symbol, gene brand and operation**

| Gene symbol | Gene brand | Operation |
|---|---|---|
| ARNTL | Aryl hydrocarbon receptor nuclear translocator-like | Circadian rhythm production is connected to TF expression. |
| BMP2 | Bone morphogenetic protein 2 | Hepcidin's upstream positive regulator |
| CYBRD1 | Duodenal cytochrome B | iron absorption from food. |
| FADS2 | Fatty acid desaturase 2 | Commonly related to changes in transcription factor expression. |
| GNPAT | Glyceronephosphate O-acyltransferase | Plasmalogens, a type of lipid, are produced by peroxisomal proteins. |
| NAT2 | N-acetyltransferase 2 | Connected to transcription factor expression associated in xenobiotic metabolism. |
| PCSK7 | Proprotein convertase subtilisin/kexin | In the fundamental secretory pathway, serine protease is related to the production of proproteins. |
| PNPLA3 | Patatin like phospholipase domain-containing protein 3 | A multi - functional enzyme that functions as both a triacylglycerol lipase and an acylglycerol O-acyltransferase in adipocytes. |
| TF | Transferrin | The most important iron transport protein in the blood |
| TMPRSS6 | Transmembrane serine protease 6 | Hepcidin's upstream negative regulator |

**Figure2**




Some people don't get any symptoms until other problems arise. These may include:

- Liver problems
- Diabetes
- Arthritis
- Abnormal Heartbeat
- Erectile Dysfunction

Different complications can arise based on the organs responsible for iron absorption. Because a huge amount of iron is stored in the liver, it's not surprising that the liver undergoes a lot of fibrosis as a result of free radical damage over time and causes liver cancer.

**Treatment of Hemochromatosis:** It usually involves phlebotomy, which is an age-old treatment for a variety of ailments. The iron load is decreased by eliminating red blood cells till the serum ferritin and percent concentration levels are reduced. Deferoxamine is a stimulant treatment that is used as a medication. Deferoxamine binds to free iron in the blood and allows it to pass through the urine, lowering the iron load once. Table3 analyses the drug molecule used for the treatment of hemochromatosis (Guggenbuhl et al. 2005; Lan et al. 2005; McDermott and Walsh 2005; Kowdley et al. 2020).

**Phlebotomy:** Phlebotomy is the preferred medication, but in the most severe cases, complementary oral chelation may be used. Phlebotomies are also effective for treating patients with defunct ferroportin disease, but it may be done on a limited basis due to the risk of anaemia in these patients due to poor iron recycling (McLaren et al., 2010). Many doctors and patients believe that high serum ferritin levels indicate iron overload and that phlebotomy is used to treat it. Phlebotomy therapy is used to take out iron from the body and avoid further tissue damage (McLaren et al. 2010). Patients with haemochromatosis should resist oral iron medication and excessive drinking, but there are no nutritional limitations. Raw shellfish should be avoided by patients with hemochromatosis, especially in subtropical areas, because they are more susceptible to Vibrio spp. Infections (Pilling et al. 2019; Kowdley et al. 2020).

**Chelation therapy:** When phlebotomies are forbidden by treatments due to inconceivable poor vein situation, iron chelator treatment is only used in exceptional and unique cases of HH (Kowdley et al. 2020).

**Table 2**

| Absorption of iron on the organ | Disorder | Result |
|---|---|---|
| liver | free radical damage over time | risk of liver cancer |
| heart muscle | Development of cardiomyopathy | Arrhythmias |
| skin | Increase in melanin | bronze-colored skin |
| Pituitary gland | Gonadal dyfunction and affect release of sex hormones | Amenorrhea in women and testicular atrophy in man |
| Joint | Calcium crystal accumulation | Degenerative joint diseases |

**Hepcidin therapies:** This strategy is best suited for standard treatment if drugs do not reduce iron in the liver. The therapies will almost certainly be parenteral and costly. For patients with haemochromatosis, the SF-36 (Short Form 36) is the most frequently applied device (Kowdley et al. 2020).

**Table 3. The drug molecule used for treatment of Hemochromatosis**

| Drug Name | Group | Brand Name | Chemical Formula | Drug Bank Accession Number | Drug Bank Link |
|---|---|---|---|---|---|
| Deferasirox | Approved, Investigational | Exjade, Jadenu | $C_{21}H_{15}N_3O_4$ | DB01609 | https://go.drugbank.com/drugs/DB01609 |
| Deferoxamine | Approved, Investigational | Desferal | $C_{25}H_{48}N_6O_8$ | DB00746 | https://go.drugbank.com/drugs/DB00746 |
| Deferiprone | Approved | Ferriprox | $C_7H_9NO_2$ | DB08826 | https://go.drugbank.com/drugs/DB08826 |
| Deferitazole | Investigational | — | $C_{18}H_{25}NO_7S$ | DB13120 | https://go.drugbank.com/drugs/DB13120 |
| Deferitrin | Investigational | — | $C_{11}H_{11}NO_4S$ | DB16132 | https://go.drugbank.com/drugs/DB16132 |
| Amlodipine | Approved | Amlobenz, Azor, Caduet | $C_{20}H_{25}C_lN_2O_5$ | DB00381 | https://go.drugbank.com/drugs/DB00381 |
| Pancrelipase | Approved, Investigational | — | — | DB00085 | https://go.drugbank.com/drugs/DB00085 |

## CONCLUSION

The findings of the present study have shown that hemochromatosis is a build-up of iron in our body and can be harmful to your liver, heart, endocrine and glands. A liver biopsy may be applied in some cases to verify the presence of iron overload. Hypogonadism, cardiomyopathy, and liver fibrosis are all common clinical features. We present mutations in the HFE gene, and transferrin saturation. We suggest complete non-coding region screening of erythrocytosis-associated genes for all statistically significant variations that may be linked to an enhanced expansion of RBCs, followed by whole-genome sequencing, to further investigate the aetiology of instances of congenital erythrocytosis.

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**Biotechnological Communication**

# Optimizing α-amylase Production from locally Isolated *Aspergillus* species Using Selected Agro Waste as Substrate

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## ABSTRACT

The purpose of this research is to determine whether it is possible to produce α-amylase from agricultural waste (groundnut shell).. The strain (M1) identified as *Aspergillus* sp. exhibited the largest clearance zone (1.6 cm) and was used in fermentation studies. The activity of α-amylase increased after 24 hours of fermentation, peaked at 72.3 U/mL on day 5, and then began to decline. The effect of optimized environmental conditions studied using OFAT, and it was discovered that pH 6, inoculum size of $1 \times 10^7$ spores/mL, incubation period of 120 h, substrate concentration of 3 percent (w/v), and temperature of 35 °C were the best for producing α-amylase from groundnut shell using *Aspergillus* sp. In a single fermentation, these optimum conditions were used, and the experiment yielded an optimum enzyme yield of 121.3 U/mL. This research shows that groundnut shell, a low-cost and commonly available waste, could be an ideal substrate for the manufacture of value-added products.

**KEY WORDS:** α-AMYLASE, OFAT, FERMENTATION, OPTIMIZATION, *ASPERGILLUS* SPECIES.

## INTRODUCTION

Groundnut (*Arachis hypogea*. L) originated in Latin America and was introduced to West Africa by Portuguese traders in the 16th century. Developing nations account for 97% of acreage and 94% of worldwide crop production. (Tela et al. 2021). Nigeria was the third largest producer of groundnuts in the world in 2021, trailing only China and India, with output values of 16,685,915, 6,857,000, and 3,028,571 tonnes respectively (Shanthala et al. 2022). Several researchers have stated that cultivating microorganisms on lignocellulosic materials is a promising strategy for creating enzyme, which will reduce the cost of producing enzyme. Lignocellulosic materials are abundant in the environment, accounting for half of all terrestrial biomass (Sabino et al. 2021) Lignocellulosic wastes are a complex structure made up primarily of cellulose, hemicellulose, and lignin, which are linked by covalent bonds to create a complex network resistant to microbial invasion. Lignocellulosic wastes are a low-cost source of enzyme production (Sabino et al. 2021). To keep lignocellulosic waste from becoming a nuisance in the environment, it has been used to make a variety of value-added goods, such as enzymes (Santana et al. 2021). Agricultural and industrial operations produce lignocellulosic waste. Sugarcane bark, bagasse and straw, rice straw and rice bran, cassava peel, maize cobs and straw, wheat chaff and bran, banana straw, cassava peel, wood scraps, and groundnut shell are only a few examples of lignocellulosic wastes produced in Nigeria (Igbokwe et al. 2022). It may interest you to learn that these plentiful wastes are underutilized, contributing to pollution problems in the environment. Bioethanol, enzymes, organic acids, biosurfactants, biogas, biohydrogen, and biofertilizers have all been developed using lignocellulosic wastes as a viable substrate due to their high nutrient content (Chen et al. 2022).

Amylases are used to hydrolyze polysaccharides like starch into simple sugar constituents in the starch processing industry. As new opportunities in biotechnology have emerged, the range of amylase applications has expanded to include analytical chemistry, medical and pharmaceutical applications. (Almulaiky, et al. 2021). Amylases are one of the most essential enzymes and are very important in biotechnology; they are a class of industrial enzymes that account for around one quarter of the global enzyme market (Kalia et al. 2021). The key benefit of using microorganisms to produce amylases is the least expensive

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of mass production as well as the ease with which microbes can be influenced to produce desired enzymes. However, the cost of generating α-amylase is so expensive, there is a need to develop more cost-effective methods of producing the enzyme. This can be accomplished by making use of widely available and abundant wastes, such as groundnut shell, which is a common solid waste in underdeveloped countries. Its ability to produce α-amylase will facilitate waste management at a low cost, minimize pollution caused by garbage, and expand the country's economic basis. The goal of this study is to produce α-amylase from cheap and readily available waste (groundnut shell) by optimizing the fermentation conditions by One Factor at a Time (OFAT). The enzyme will be produced by locally isolated *Aspergillus* sp. isolated from soil samples.

## MATERIAL AND METHODS

Groundnut shells were collected from local farmers in Kano State, Nigeria, then crushed into fine powder in a milling machine. The glassware which include test tubes, MacCartney bottle, beakers, conical flask and measuring cylinder were washed with detergent and rinsed thoroughly with water. They were allowed to dry, wrapped in aluminum foil and then sterilized at temperature of 160 ˚C for 60 minutes. The inoculating loop and cork-borer were sterilized by dipping in flame until they were red hot. The spatula was sterilized using alcohol and bent glass rod was sterilized with alcohol and flame (Pasin et al. 2020).

Potato Dextrose Agar was prepared following the manufacturer's specification while starch agar was prepared following the method described by (Saha and Mazumdar, 2019). The media were sterilized at 121ºC for 15 minutes. Soil samples were used to isolate amylase-producing fungus. The soil samples were taken at a landfill site for cassava garbage. Before plating on Potato Dextrose Agar using the pour plate technique, the samples were serially diluted to a concentration of 10-4. The fungal plates were incubated for 3-7 days at 30°C. Isolates with unique clusters were carefully selected after incubation and sub-cultured on fresh media to obtain pure culture. At 4 ºC, pure cultures were kept in agar slants (Pasin et al. 2020).

All fungi isolates were identified conventionally. Microscopic and macroscopic views of the isolates were used for identification. The colonial morphology of the fungal isolates on the plates was used to identify them. Cultural and morphological factors such as the nature of the hyphae, color of the colonies, appearance of the colonies and growth rates were taken into account for proper characterization of the isolates, as described by (Ani et al. 2021). This involved using a sterile needle to pick a small bit of mycelial mat and placing it on a clean glass slide, staining with lacto-phenol cotton-blue, and covering with a cover slip. There were reproductive and vegetative structures found. During microscopy, the type of spores, sporangia, hyphae branching, and the presence of septa were all considered. A fungal atlas was used to identify the isolates (Ani et al. 2021).

The best isolate used for the production of fungal amylase was being identified at Centre for Biotechnology Research Kano, Kano, Nigeria. The methods used were the sequencing of ITS1 and ITS2 regions of the genomic DNA, followed by comparison of sequence similarity with other fungi on the National Centre for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Screening was performed on the isolated organisms using the method outlined by (Saha and Mazumdar 2019). Peptone, 0.90 g/L; $(NH_4)_2HPO_4$, 0.40 g/L; KCl, 0.10 g/L; $MgSO_4$,$7H_2O$, 0.10 g/L; starch soluble, 10 g/L and 2 percent (w/v) agar-agar were included in the amylase agar. The isolates were streaked on amylase agar and cultured for 7 days at 30 ºC. After incubation, the plates were filled with iodine solution and incubated for another 30 minutes. After that, the plates were rinsed with double distilled water and looked for a starch hydrolysis zone of clearance around the colony expansion. As amylase producers, microbial colonies with the highest zone of clearance were chosen.

Using an inoculating loop, spores from 7-day-old fungal cultures were scraped and aseptically transferred to sterile distilled water comprising 0.1 percent Tween-80 as described by El-Ghonemy (2021). To optimize the inoculum size, incubation duration, pH, temperature and substrate concentration, One Factor at a Time (OFAT) was employed. To modify the appropriate moisture level, the fermentation media contained 2 mL of mineral salts solution comprising of 2g potassium dihydrogen phosphate ($KH_2PO_4$), 5g ammonium nitrate ($NH_4NO_3$), 1g sodium chloride (NaCl) and 1g magnesium dihydrogen sulfate ($MgSO_4.7H_2O$) in a liter of distilled water. All the ingredients were combined, autoclaved for 20 minutes at 121°C, then allowed to cool. After that, spore suspensions were placed on a sterile solid substrate and incubated at different temperatures (El-Ghoney 2021).

Temperature, incubation time, pH, inoculum size and substrate concentration are all key factors in enzyme development and have a significant impact on enzyme activity. The optimization experiments were conducted using one factor analysis with three replicates for each determination according to Batista et al. (2021). Fermentation was observed for 7 days at 30 ºC. After 24, 48, 72, 96, 120, 144, and 168 hours of incubation, samples were taken to determine amylase activity. The temperature for incubation was set within the range of 30, 35, and 40 ºC for the selection of the optimum temperature for amylase production, and the amylase test was performed at the end of the optimum incubation period. Using a pH meter, the pH of the fermenter was modified to several levels ranging from (3.0, 4.0, 5.0, 6.0 and 7.0) using two buffer systems (1N NaOH and 1N HCl). The amylase assay was performed at the previously determined optimum time. Each fermentation medium was fed with varying inoculum sizes ($1 \times 10^5$ and $1 \times 10^7$ spores/mL). Amylase activity was assayed at the end of the optimum incubation period.

Different concentrations of the substrates (groundnut shell) at 1 %, 2 %, 3 %, 4 %, and 5 % (w/v) were used in separate fermentation flask. Amylase activity was assayed at the end of the optimum incubation period. The fermented quantity

was combined with 50 mL distilled water at the end of fermentation and stirred for 1 hour on a rotary shaker at 150 rpm. After filtering through Whatman filter paper No. 1, it was centrifuged for 15 minutes at 6000 rpm. The cell-free supernatant was extracted as crude enzyme for further investigation (Olakusehin et al. 2021).

According to Miller's DNSA assay method, the amount of reducing sugars produced in a mixture containing 1.0 mL soluble starch in phosphate buffer, pH 6.0, and 1.0 mL enzyme extract was measured to determine α-amylase activity (Deshayath et al. 2020). After 10 minutes of incubation in a water bath at 50°C, the reaction was stopped with 1.0 mL of dinitrosalicylic acid (DNSA) reagent and the mixture boiled for 15 minutes. After allowing the test tubes to cool, the absorbance was measured at 540 nm with a UV spectrophotometer. The glucose concentration discharged was compared to a glucose standard. Under assay conditions, one enzyme activity unit (U) was described as the quantity of enzyme that released one mole of reducing sugar per minute per milliliter.

## RESULTS AND DISCUSSION

The results of this work aimed at optimizing alpha amylase production from locally isolated *Aspergillus* sp. using agricultural waste (groundnut shell) as substrate are documented in the subsections below:

**Isolation and characterization of fungal isolates:** Six fungi were isolated from the soil samples and were given the letters M1, M2, M3, M4, M5, and M6. Table 2 lists the macroscopic and microscopic properties of the isolated fungi, whereas Plates 1-6 depict a microscopic perspective of their vegetative structure.

**Table 1. Macroscopic and microscopic characteristics of isolated fungi**

| Isolate Code | Colonial Characteristics | Morphological Characteristic Under Microscope | Identity |
|---|---|---|---|
| M1 | Filamentous with white hyphae, production of black spores was observed on the plate after 72hrs. The reverse of the plate was brown. | Conidiophores were hyaline, erect, simple, thick-walled, enlarged at the apex, forming globose vesicles containing catenulate conidia with conidial heads | *Aspergillus niger* |
| M2 | Growth was rapid and filled the plate completely within a few days. Colonies were whitish. Dense and cottony which became greyish-brown with age, due to brownish sporangio-spores and brown black sporangia. Mycelia were interwoven | Well-developed hyphae, branched freely, coenocytic. Brown colored, smooth walled. Non-septate and erect sporangiophores developed from the hyphae | *Rhizopus stolonifer* |
| M3 | Pin like green growth. | Non-Branched conidiophore with bulb end carried conidia. | *Aspergillus flavus* |
| M4 | Yellow-orange, ochraceous, or buff colonies with restricted growth. | Non-dense colonies, sporulated, amber-colored, flaky texture, white mycelium with yellow to pale orange or gray gold reverse. Strong presence of light brown sclerotia | *Aspergillus ochraceus* |
| M5 | Colonies showed slow growth, primarily olivaceous-brown to blackish brown, but also brown, grey or buff, suede-like to floccose, and frequently powdery due to abundant conidia production. | Conidiophores were erect, straight or flexuous, unbranched or branched only in the apical region and elongated with geniculate sympodial elongation in some species. | *Cladosporium* sp. |
| M6 | Colonies were rapidly growing, white, flat, to cream in dry, color and finely suede-like, with no contrary pigment. | The holoarthric fragmentation of undifferentiated hyphae produces chains of hyaline, one-celled, smooth, sub-globose to cylindrical, slimy arthroconidia (ameroconidia). | *Geotrichum candidum* |

**Screening of isolated fungi for α-amylase activity:** Three out of the six isolates exhibited obvious zones of clearance on the amylase agar with the highest (greater than 1 cm) occurring in isolates M1 (1.6 cm) and M4 (1.2 cm) which significantly differ from others, as shown in Table 3. The isolate (M1) with the highest zone of clearance was therefore selected for further studies like molecular investigation to confirm the authentication of its identity as shown in Table 4.

Using locally isolated *Aspergillus* sp., the current study attempts to optimize α-amylase synthesis from groundnut shell. The findings of this study demonstrated that Aspergillus sp. could produce α-amylase from the substrate (groundnut shell). Six (6) distinct fungal isolates were obtained from various samples (Table 2). The isolates' morphological and microscopic properties were investigated and reported (Table 2). After that, the isolates were tested for α-amylase activity to see if they could generate the enzyme. The zone of clearance displayed by the different isolates was evaluated using amylase agar, as described in the screening of amylase producing microorganisms' section above. During the hydrolysis test, the observed zone of clearance revealed that isolate M1 had the largest

diameter of 1.6 cm, which was larger than the other isolates (Table 3). The clearance zones created around the colonies suggest that the fungal isolates can produce extracellular amylase. The findings of this investigation corroborate those of Olakusehin et al. (2021), who claimed that *Aspergillus flavus* S2-OY has amylolytic characteristics. According to Sahnoun et al. (2015), *Aspergillus oryzae* S2 has amylolytic characteristics.

**Figure 1: Microscopic view of the vegetative structure of *Aspergillus niger* (x 40)**

**Figure 2: Microscopic view of the structure of *Rhizopus stolonifer* (x 40)**

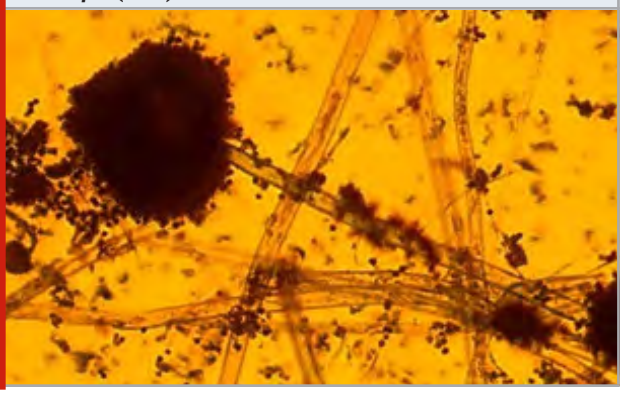


**Figure 3: Microscopic view of the vegetative structure of *Aspergillus ochraceus* (x 40)**

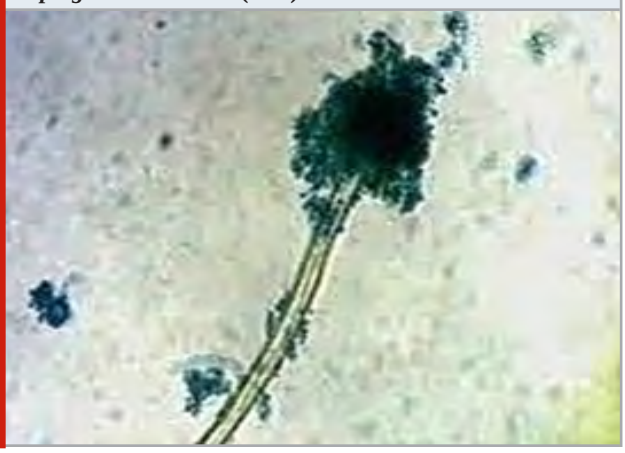


**Figure 4: Microscopic view of the vegetative structure of *Aspergillus flavus* (x 40)**

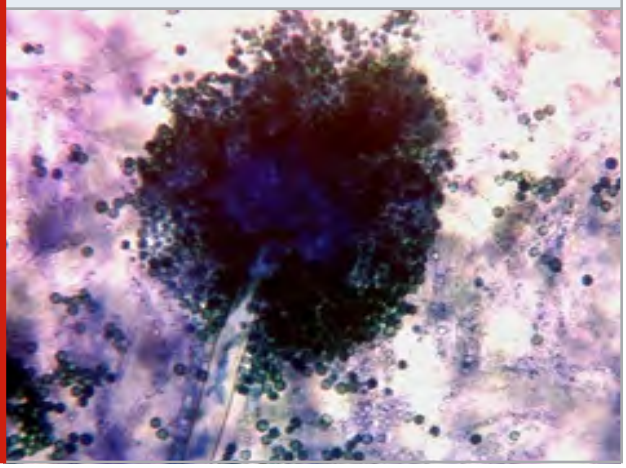
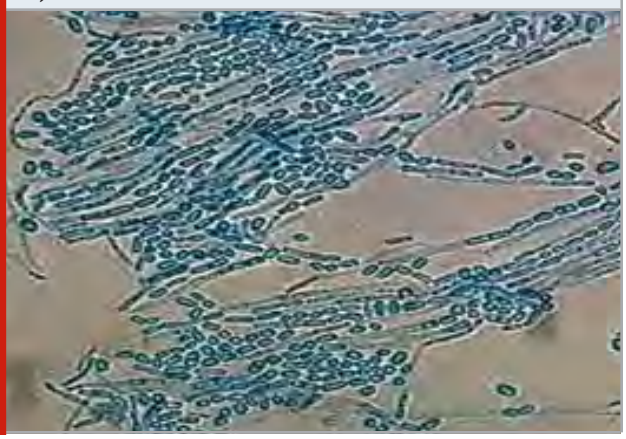


**Figure 5: Microscopic view of the structure *Cladosporium* sp. (x 40)**

**Figure 6: Microscopic view of *Geotrichum candidum* (x 40)**



Further research was conducted using isolate M1 (*Aspergillus* sp.). The isolate was used to perform time course fermentation under non-optimized conditions of pH 7.0, 2 % substrate concentration, and 1 $\times 10^7$ spore/mL inoculum size. The results showed that α-amylase activity peaked at 72.3 U/mL on day 5, with a maximum value of 72.3 U/mL. Using *Aspergillus* sp., a time course

fermentation was used to determine and monitor the trend in α-amylase synthesis from groundnut shell. The recoded enzyme yield is quite similar to that published by (Ahmed et al. 2019), who reported amylase activity of 72.4 U/mL after optimizing with OFAT (Ahmed et al. 2019).

**Table 3. Zone of clearance (cm) of different fungal isolates**

| Isolate code | Tentative name | Zone of clearance (cm) |
|---|---|---|
| M1 | *Aspergillus niger* | 1.6 |
| M2 | *Rhizopus stolonifera* | 0.5 |
| M3 | *Aspergillus ochraceus* | 1.0 |
| M4 | *Aspergillus flavus* | 1.2 |
| M5 | *Cladosporium* spp. | 0.7 |
| M6 | *Geotrichum candidum* | |

**Table 3. Molecular confirmation of M1**

| Isolate | Organism | Number of Bases | Identity | Accession number |
|---|---|---|---|---|
| M1 | *Aspergillus niger* ATCC 16888 | 576 | 100.0 % | NR_111348.1 |

**Figure 2: Shows the influence of varied incubation periods (days 1-7) on α-amylase production in *Aspergillus* sp. On day 5, the maximum enzyme activity (72.3 U/mL) was detected.**

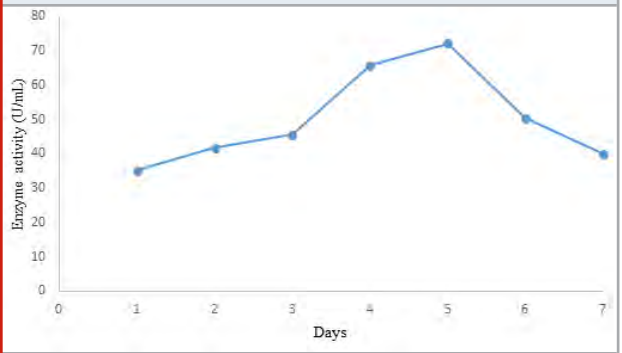



Figure 2: Trend in α-amylase production from groundnut shell using *Aspergillus* sp. Values are means ± SD; n=3

**Table 4. Effect of varying temperature (30, 35, 40 $^0$C) on α-amylase production**

| Temperature ($^o$C) | Enzyme activity (U/mL) |
|---|---|
| 30 | 65.2 |
| 35 | 88.2 |
| 40 | 71.3 |

The impact of numerous parameters was explored using OFAT, which was used to optimize a wide variety of factors and values. The impact of several parameters several parameters were investigated including pH, incubation time, temperature, substrate concentration and inoculum size. The best variables for α-amylase synthesis were pH 6, inoculum size of 1 × $10^7$ spores/mL, incubation length of 120 h, substrate concentration of 3 percent (w/v), and temperature of 35 $^o$C, as determined by an optimization experiment. In a single fermentation, these optimum conditions were used, and the experiment yielded an optimum enzyme yield of 121.3 U/mL. When compared to non-optimized settings, the enzyme activity obtained after optimization was 40 % higher. This is consistent with the findings of (Ahmed et al. 2019), who found amylase activity of 145.4 U/mL after a Response Surface Methodology optimization trial (RSM). The findings of this study shows that groundnut shell can be used to produce value-added products like α-amylase (El-Ghonemy 2021).

**Table 5. Effect of varying pH (3.0 - 7.0) on α-amylase production**

| PH | Enzyme activity (U/mL) |
|---|---|
| 3 | 63.9 |
| 4 | 66.0 |
| 5 | 72.7 |
| 6 | 87.3 |
| 7 | 79.6 |

**Table 6. Effect of varying inoculum size (1 × $10^5$ and 1 × $10^7$ spores/mL) on the total α-amylase production**

| Inoculum size (spores/mL) | Enzyme activity (U/mL) |
|---|---|
| 1 × $10^5$ | 65.6 |
| 1 × $10^7$ | 73.9 |

**Table 7. Effect of varying substrate concentration (1 % - 5 % w/v) on α-amylase production**

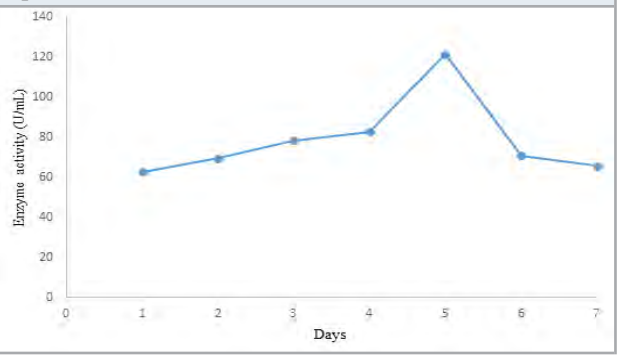
| Substrate concentration (%) | Enzyme activity (U/mL) |
|---|---|
| 1 | 57.2 |
| 2 | 59.3 |
| 3 | 65.1 |
| 4 | 54.9 |
| 5 | 52.3 |

**α-amylase production by *Aspergillus* sp.:** Time course fermentation: The result of the pre-optimization experiment under the initial conditions of growth (pH 7.0, 2 % substrate concentration and inoculum size of 1 × 107 spores/mL) is shown in Figure 2. It was observed that α-amylase activity

picked after 24 h of fermentation reaching its peak (72.3 U/mL) at day 5 before declining (Figure 2).

**Effect of optimized environmental condition, substrate, and inoculum size on α-amylase production by *Aspergillus* sp.:** Figure 2 shows the influence of varied incubation periods (days 1-7) on α-amylase production in Aspergillus sp. On day 5, the maximum enzyme activity (72.3 U/mL) was detected.

**Figure 3:Trend in α-amylase production using set of optimum conditions**




**Glucose standard curve**

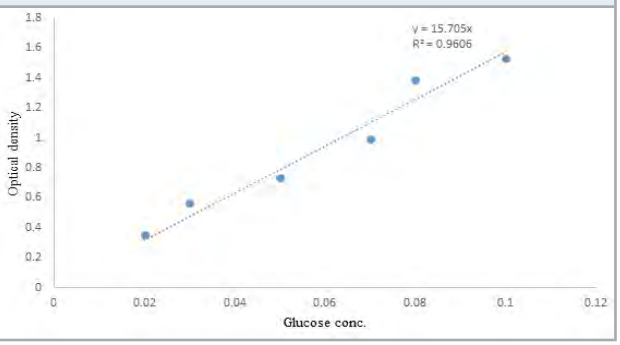



Table 4 shows the effect of temperature (30, 35, and 40 degrees Celsius) on α-amylase production. At 35 degrees Celsius, the greatest enzyme activity was (88.2 U/mL).

Table 5 shows the effect of changing pH (3.0 - 7.0) on α-amylase production. At pH 6, the maximum enzyme production was found (87.3 U/mL).

Table 6 shows the effect of different inoculum sizes (1 × 105 and 1 × $10^7$ spores/mL) on total α-amylase production. Using a 1 × $10^7$ spores/mL inoculum size, the greatest enzyme production was found at (73.9 U/mL).

Table 7 shows the effect of different substrate concentrations (1 % - 5 % w/v) on α-amylase production. At a substrate concentration of 3 % w/v, the greatest enzyme production was obtained (65.1 U/mL).

**α-amylase production using optimum conditions:** The set of optimum conditions obtained from this study were combined in a single fermentation and the result is shown in Figure 3.

## CONCLUSION

The findings of the present study have shown that in non-optimized experiments, the maximum α-amylase concentration was 72.3 U/mL, while the optimum concentration was 121.3 U/mL after optimization with the optimum conditions of pH 6, inoculum size of 1x$10^7$ spores/mL, incubation period of 120 h, substrate concentration of 3% (w/v), and temperature of 35 °C. The use of groundnut shell as an enzyme substrate proved to be highly promising, with reasonable yields. As a result, these findings imply that groundnut shell, which is typically regarded as a waste, might be exploited as a cheap agro-industrial substrate for enzyme production.

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# Postpartum Depression among Women in Kerala: A Survey

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## ABSTRACT

The increase in number of cases of Postpartum Depression (PPD) in Kerala increases day by day. A study on the awareness of PPD is necessary for this scenario. This study tries to quantify the awareness women in Kerala have about PPD and it also covers how they tackled the issue – various methods used by them to cope with the issue. The study is conducted using an online survey method. A prepared questionnaire is circulated online among 150 young mothers who were born and brought up in Kerala. The questionnaire consists of 8 questions about PPD and baby blues. Each question is provided with options from which the participants can choose one. The data thus collected are studied and analysed. The results of the survey are analysed to arrive at a conclusion. There was active participation from the side of the participants. The results show that the percentage of participants who are well aware of PPD is very low. The study brought to light that much attention and activities are needed in the case of PPD in Kerala. Most women who suffer from PPD are reluctant to seek medical help. This situation has to be changed through proper campaigns and other related activities.

**KEY WORDS:** ANXIETY, AWARENESS, COPING MECHANISM, POSTPARTUM DEPRESSION, TREATMENT.

## INTRODUCTION

Postpartum Depression is a serious issue that is faced by many women in present-day society. Postpartum Depression (PPD) is a medical condition that can be cured with proper care and treatment. In women, depression can occur during and after pregnancy. Depression after delivery can occur as "baby blues" that last only for one or two weeks after childbirth. It has mild symptoms like mood swings, anxiety, and insomnia. A more severe condition is Postpartum Depression. It is long-lasting than baby blues and shows intense symptoms including anxiety and panic attacks, sadness, irritability, severe mood swings, problems in appetite, difficulty in bonding with your baby, thoughts about harming baby or yourself and severe anger (Jayarajan 2021).

The lack of awareness about Postpartum Depression increases the depth of the problem. Studies show that the lack of early detection of PPD also worsens the condition. The awareness of the real problem – that is, PPD is a serious issue that can affect the female's later life – can bring some changes in the present situation (Zauderer 2009). The purpose of the study is to bring the problem of PPD to the forefront and thus reduce the risk women face nowadays. Such a study is relevant where people are unaware of the seriousness of PPD and when no proper care is given to women suffering from PPD. The study tries to quantify women's awareness of the issue (Jayarajan 2021). The relevance of the study is high as there are no such studies conducted in Kerala in recent times. Moreover, a study like this can contribute to the area of mental health, the overall well-being of a society and the development of the health sector of Kerala.

## MATERIAL AND METHODS

An in-depth analysis of Postpartum Depression was conducted using a survey. The study was conducted by circulating the prepared questionnaire among 150 young mothers. The sample of the study was selected after much research on the topic. The ages of the participants were in the range of 25 – 40. Mothers who gave birth in the last 10 years, and who were born and brought up in Kerala were considered for this study.

This was done to examine the recent developments in the area, especially in Kerala. The research subjects were verified to be cognizant of English and all the questions were in English. The participants were well informed about the intention of the survey. They were informed to read the instructions clearly and answer the questions. It is also assured that their details will be kept confidential. The survey mainly aimed at checking the knowledge/awareness women in Kerala had about PPD. Moreover, the study checked how they came to know about PPD and how far they were aware

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of the issue. It also helped to collect information about their personal experience of PPD, and how they overcame it. The problem of bonding with the baby was also included as one question.

The questionnaire consisted of 8 questions about PPD and baby blues. These were intended to collect information about the level of awareness women had about PPD, how they came to know about it, have they attended any awareness programs etc. The survey also tried to collect information about the coping mechanism the participants chose to overcome the situation. Responses to this particular question revealed how worse are the condition and what percentage of women seeks medical help. There were questions about the symptoms they had to suffer and about the time they experienced it. Each of the questions was given options from which the participants can choose one. The number of the options varied from question to question, that is, from 2 – 5. The research subjects were verified to be cognizant of English and all the questions were in English.

The participants were well informed about the intention of the survey. They were informed to read the instructions clearly and answer the questions. It is also assured that their details will be kept confidential (Jayarajan 2021). The present study has been approved by the Institutional Ethics Committee of Amrita Vishwa Vidyapeetham, Coimbatore. All due permissions have been taken by the concerned authorities including consent etc.

## RESULT AND DISCUSSION

The intensity of the psychic problems faced by women suffering from PPD needs to be discussed in detail, and a long-lasting solution has to be found. An important part of reducing the percentage of patients can be done by giving proper awareness. Not only females but also males and people of other genders have to be educated about the mental condition of women during and after pregnancy. Medical professionals suggest providing proper awareness can reduce the problem in a considerable way. This can contribute a lot to the healthy development of the baby and mother (Zauderer 2009).

**Figure 1: Pie chart showing the awareness of participants about Baby Blues**

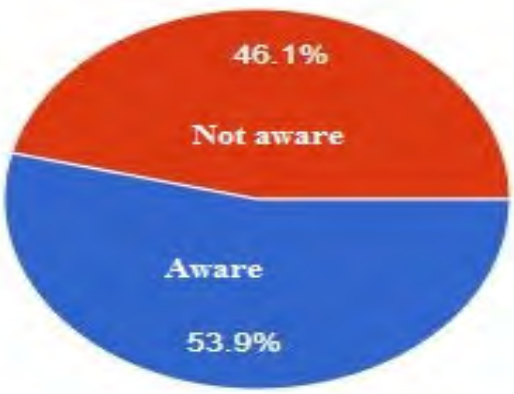


**Figure 2: Pie chart showing the awareness of participants about PPD**

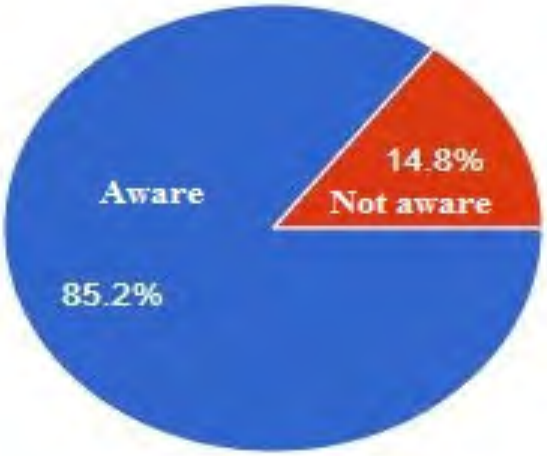


Figure one shows that only about 53.9% of the participants heard about baby blues. This revealed the intensity of the situation. The participants included in the other section, that was, the 46.1%, might be the ones who have gone through the same. But unawareness of the real problem worsens the condition. As baby blues last only for two or three weeks it is not a dangerous problem as PPD. But even in the case of PPD, a considerable percentage of people who were unaware of PPD was found. Figure 2 shows that 14.8 % of the participants were unaware of postpartum depression. And the results show that people were not much aware of serious problems like PPD as they are of Baby Blues. This condition has to be changed through continuous practices to make the general public aware of PPD and its symptoms (Jayarajan 2021).

**Figure 3: Pie chart showing the response of participants to whether they faced symptoms of PPD**

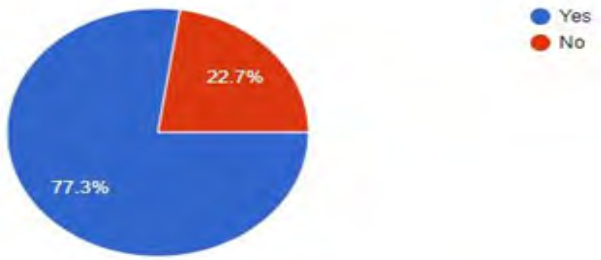


**Figure 4: Pie chart showing how much time did the participants felt the symptoms**

There exists a social stigma in India, especially in Kerala, to consult a psychiatrist or a psychologist (The Hindu 2018). This is another reason which prevents the cure of PPD through proper treatment. People are reluctant to admit the fact that they are facing some mental problems. The same kind of reluctance is there in the case of PPD also. They fear a kind of 'Othering' from society and its roots can be traced back to the stigma toward a madness that existed in the Middle Ages. This tendency limits the possibility to take proper medical care. This can also be controlled by conducting awareness programs and thus normalizing mental problems and depression (Foucault 1988; Kuriakose et al. 2020; Jayarajan 2021).

Figure three and four brings out the seriousness of the situation. In Figure three, one can see that about 77.3% of participants faced the symptoms of PPD. This is proof of the problem faced by women after delivery. Figure four gives the period for which they faced these difficulties. 19.2% faced symptoms of PPD up to one year after delivery/C-section. For 28.3% it lasted for six months, and for 24.2% it lasted up to one month. Only 28.3% experienced it for two weeks after childbirth (Kuriakose et al. 2020).

**Figure 5: Pie chart showing the level of difficulty the participants faced in bonding with the baby**

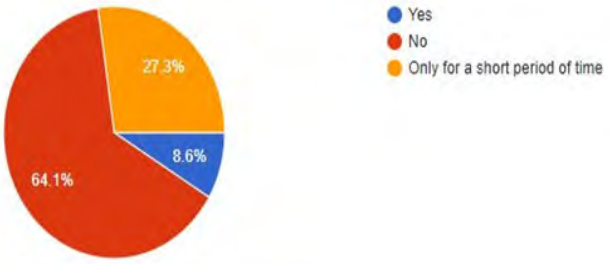


**Figure 6: Pie chart showing how participants with PPD cope up with it**

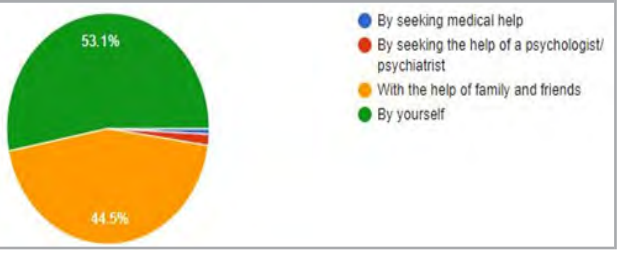


Figure 5 shows that 8.6% of the participants found it difficult to create a bond with the baby and 27.3% of them felt the same for a short period. This is a period that is highly dangerous and may lead up to harming a child and oneself. Recent news reports give evidence of this were mothers in their depressive state harmed babies and some violent acts ended up in their death (Jayarajan 2021). The results of the survey indicate that most of the participants have gone through severe Postpartum Depression and all of them needed medical help. But Figure 6 shows the real scenario of Kerala's treatment of a serious mental issue like PPD (Jayachandran 2021).

Even though most of the participants faced severe PPD-related problems only 0.8% of them sought medical help and only 1.6% of them were ready to seek the help of a psychologist or psychiatrist. This reveals the pathetic condition of Kerala concerning PPD. This situation can be changed by giving proper awareness about the issue. The collected data shows that only 7% of the participants had attended any awareness programs related to PPD. Most of them learned about this issue through social media. And some of them learned through articles and only 1.7% of them got informed through the newspapers. This shows that the government, health department, as well as the public, have to play a significant role in reducing the difficulties faced by mothers and newborns (Kuriakose et al. 2020).

The study,'Prevalence of depression among middle-aged women in the rural area of Kerala', conducted by Archana P S, Soumitra Das and Sairu Philip et al., tries to determine the prevalence of depression among women aged 40 – 60 years in the rural area of Kerala using Patient Health Questionnaire 9 (PHQ – 9). The setting of the study is Ambalappuzha, a rural area in the district of Alappuzha. (Archana et al. 2017; Kuriakose et al. 2020). In contrast, this study tried to include young women from all over Kerala who gave birth in the last 10 years. Moreover, this study concentrates only on depression that comes as a result of pregnancy and/or delivery.

## CONCLUSION

The findings of the present study quantify the awareness young mothers in Kerala have about PPD. As this study deals with a health-related issue which is related to the well-being of society, it has significance in the present scenario. This study prompts studies in the future that may lead to change in the current situation. Proper awareness about the issue can be given through newspapers, news channels, social media and other media. Constant active participation from the public and initiatives from the government is needed to improve the situation.

## ACKNOWLEDGEMENTS

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**Conflict of Interests:** Authors declare no conflict of interests to disclose.

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# Stability Indicating Reverse Phase High-Performance Liquid Chromatography Method for Quantitative Estimation of Impurities in Gadobutrol Solution for Intravenous Administration

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## ABSTRACT

The purpose of this investigation was to establish a HPLC methodology and evaluate Gadobutrol along with impurities A, B and C. The method for Gadobutrol along with impurity-A, B and C analysis was developed on Phenomenex Phenyl-Hexyl C18 column with isocratic elution using mobile phase consisted of formic acid (pH 3.6) and acetonitrile. The method proposed showed a good linearity in the range of 0.8314 – 30.21 ppm (Gadobutrol), 3.2971 – 34.62 ppm (impurity-A), 0.3788 – 34.82 ppm (impurity-B), and 2.9757 – 32.38 ppm for impurity-C. Obtained good method precision (RSD = 2.36% to 3.55%), acceptable accuracy (98.8% to 108.9%), detection limit (0.1250 ppm to 1.0811 ppm) and quantitation limit (0.3788 ppm to 3.2971 ppm) for Gadobutrol and impurity-A, B and C. The method proposed can be utilized to assess the quality of Gadobutrol sample for the presence of impurities A, B and C.

**KEY WORDS:** GADOBUTROL, RELATED SUBSTANCES, RP-HPLC, STABILITY INDICATING, UV DETECTOR, VALIDATION.

## INTRODUCTION

Effective evaluation of process associated impurities is crucial for the development of pharmaceuticals. Process associated impurities are related to the production process and may include precursor substrates, intermediates formed during the procedure of synthesis, solvents used during production or purification (Jinshu et al. 2015; Kung-Tien and Chien-Hsin 2019). As mentioned by the "International Conference on Harmonization" for the qualification of the drug substance, the permissible level for known process associated impurity needs to be <0.15% and the unknown process associated impurity need to be <0.10% (ICH Q3B(R2) 2006; ICH Q3A (R2) 2008). In order to comply with the strict regulatory necessities, it is necessary to identify impurities and carefully monitor their amounts. Gadobutrol is utilised in adult as well as pediatric sufferers, including newborns, with magnetic resonance imaging to diagnose and predict areas with an impaired blood barrier and/or aberrant central nervous system vascularity (Scott 2018; Glutig et al. 2019; Petronek et al. 2021; Akbas et al. 2022).

The 1, 4, 7, 10-tetraazacyclododecane is used to make the Gadobutrol pharmaceutical substance. Impurities impurity-A, B and C, as well as two other molecules, are produced as by-products during the manufacturing of Gadobutrol. Purification removes the generated two additional compounds, and remaining impurities (A, B, and C) are regulated in the drug material. Impurity-A was designated chemically as 2,2′-[4,10-bis[2,3-dihydroxy-1-(hydroxymethyl)propyl]-1,4,7,10-tetra azacyclododecane-1,7-diyl]diaceticacid (Figure 1). Impurity-B was designated chemically as 2,2′,2″-[10-[(1RS, 2SR)-2,3-dihydroxy-1-(hydroxymethyl)propyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triyl]triacetic acid (Figure 1). Impurity-C was designated chemically as gadolinium 2,2′,2″-(1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetate (Figure 1) (Akbas et al. 2022).

Also, after so many purification phases, impurities impurity-A, B and C will often occur which canco-purify

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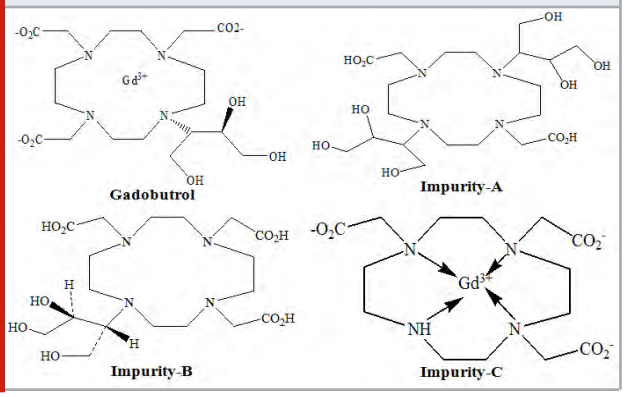
with the Gadobutrol. The existence of impurity-A, B and C impurities in Gadobutrol can potentially influence the efficacy of the final Gadobutrol drug. Therefore, a sensitive, reliable and effective method is required to detect and evaluate impurities A, B and C in Gadobutrol. The specification thresholds for impurities A, B and C impurities were considered as 0.20 % concentration based on new drug substances policy given in ICH Q3A (ICH Q3A (R2) 2008; Al-Sabti and Harbali 2021; Kalauz and Kapui 2022). Detection and evaluation of impurities A, B and C impurities in the Gadobutrol using any analytical approach is not yet documented. Therefore, a sensitive, reliable and effective HPLC dependent method to detect and evaluate impurities A, B and C impurities simultaneously in Gadobutrol was developed and validated herein.

**Figure 1: Structures of Gadobutrol and impurities A, B and C**




## MATERIAL AND METHODS

HPLC grade Merck (Mumbai, India) formic acid, acetonitrile, water- HPLC-grade (Milli Q) were utilized. Impurity-A (85.7% purity), Impurity-B (94.7% purity) and Impurity-C (90.03% purity) were obtained from Aavyan Labs Pvt Ltd. (Hyderabad, India). Jodas Expoim Pvt Ltd. (Hyderabad, India) provided Gadobutrol. Agilent (USA) HPLC 1260 infinity system, Agilent (USA) photodiode array detector, Open lab software, Sartorius MSA6.6S-000-DM microbalance, Bandelin, DT514BH ultra sonicator and Polmon LP-139SA pH meter were used during detection and evaluation of impurity-A, B and C impurities simultaneously in Gadobutrol.

A Phenomenex Luna Phenyl-Hexyl C18, 250 × 4.6 mm, 5 µm column was used at 50 °C temperature with isocratic elution at the rate of flow with 1 mL per min. The mobile phase contained of aqueous formic acid (pH 3.6) and acetonitrile in 95.5% volume and 0.5% volume, respectively. Sample quantity of 20 µL, sample injector temperature of 5 oC and wavelength of 195 nm were utilized in the detection besides evaluation of impurities A, B and C impurities in the Gadobutrol. Mixed stock solution (2.0% concentration) for Gadobutrol and impurities A, B and C was prepared in mobile phase. Series of working solutions (LOQ to 150% of Gadobutrol, impurities A, B and C specification value limits) were prepared by diluting appropriate aliquots of the mixed stock solution (2.0% concentration) with mobile phase. Working solution of Gadobutrol and impurities A, B and C with 20 ppm concentration were also done by diluting appropriate aliquots of stock solution (2.0% concentration) with mobile phase.

This solution with concentration 1 mg/mL was prepared in mobile phase. The Gadobutrol sample was shaken for 5 min after the preparation and filtered using Millipore 0.45 microns. Accurate quantities equal to 0.8314 – 30.21 ppm of Gadobutrol, 3.2971 – 34.62 ppm of impurity-A, 0.3788 – 34.82 ppm of impurity-B, and 2.9757 – 32.38 ppm of impurity-C were prepared. Each quantity solution was infused (20 µL) and recorded the corresponding chromatograms and peak areas for Gadobutrol, impurity-A, B and C by applying suggested HPLC method. The calibration curves for each component (Gadobutrol, impurity-A, B and C) were then generated utilizing its respective peak areas, and corresponding regression linear equations was then created.

The Gadobutrol solution was infused (20 µL) and noted the chromatograms by applying recommended HPLC method. The response areas of impurities A, B and C in Gadobutrol solution were documented. The percentage of impurities A, B and C in Gadobutrol solution was assessed using corresponding regression linear equations. Gadobutrol was subjected to a number of forced degradation tests, involving acid, base, humidity, peroxide, humidity and thermal degradation [ICH Q1A (R2); 2003]. Gadobutrol was subjected to acid made hydrolysis (1.0 N HCl, 60 °C over 1 hr), alkali made hydrolysis (1.0 N NaOH, 60 °C over 1 hr), peroxide made oxidation (bench top 4 hr), thermal made degradation (105 °C, over 24 hr) and humidity made degradation (90% humidity, over 24 hr). The degraded Gadobutrol solutions were infused (20 µL) and noted the chromatograms by applying the suggested HPLC method. The occurrence of impurities A, B and C impurities and percent degradation of Gadobutrol were documented.

## RESULTS AND DISCUSSION

Method establishment: Zorbax SB C8, 250 × 4.6 mm, 5 µm column and Phenomenex Luna Phenyl-Hexyl 250 x 4.6 mm, 5 µm columns with isocratic elution with solvents combination of 0.1% aqueous formic acid: acetonitrile with different pH values and different ratio volumes were tried during trail experiments. During trails, sample volume size for analysis, column temperature, sample port temperature and flow rate are kept constant at 20 µl, 50 °C, 5 °C and 1.0 mL/min, respectively. Based on resolution, peak shape and sensitivity values obtained during trails (Figure 2), Phenomenex Luna Phenyl-Hexyl 250 × 4.6 mm, 5 µm column with isocratic elution with 0.1% aqueous formic acid and acetonitrile in 95.5% volume and 0.5% volume, respectively, as mobile phase was opted as optimal conditions to detect and evaluate A, B and C impurities simultaneously in the Gadobutrol sample. The wavelength of choice for the study of A, B and C impurities was 195 nm, where impurities showed more sensitivity.

**Validation:** The method for A, B and C impurities evaluation in the Gadobutrol was verified in harmony thru ICH strategies (ICH Q2 (R1) 2005).

**Figure 2: Gadobutrol drug and impurities A, B and C chromatogram**

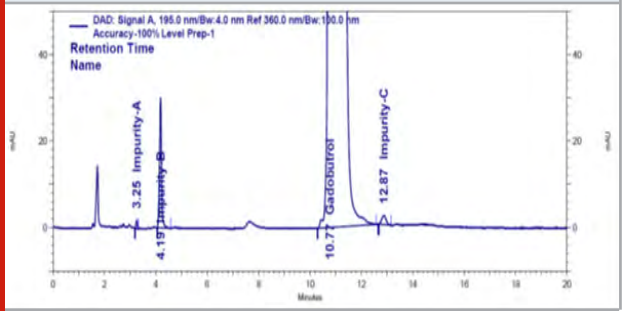



**Figure 3a: Diluent chromatogram**

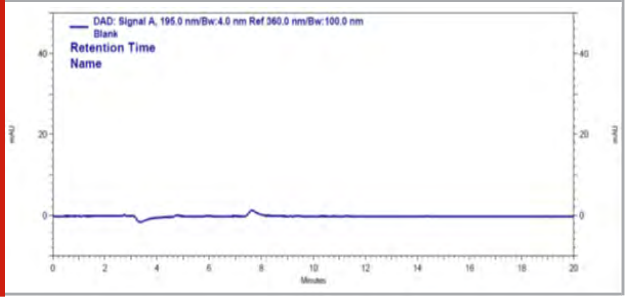



**Figure 3c: Impurities A, B and C spiked Gadobutrol sample chromatogram**

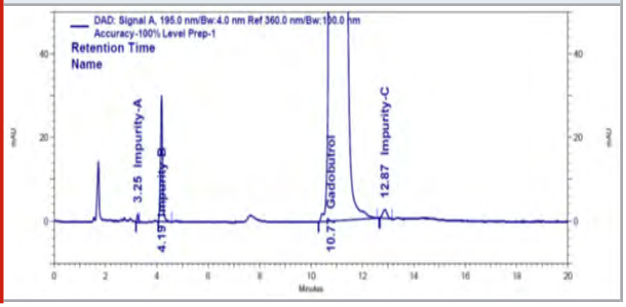
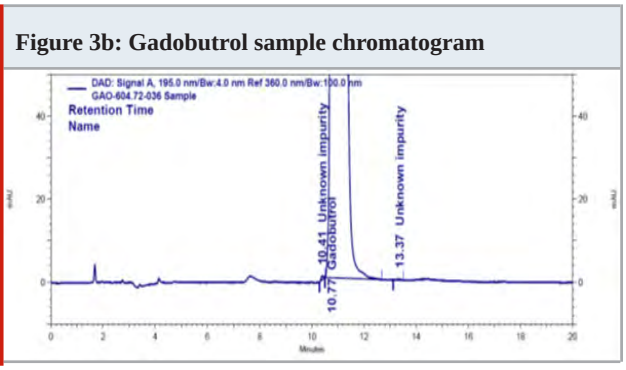



**Figure 3b: Gadobutrol sample chromatogram**




**Specificity:** The procedure's specificity was tested to ensure that the Gadobutrol and diluent components did not interfere with the analysis of A, B and C impurities. Gadobutrol sample, Gadobutrol solution spiked with impurities A, B, and C (20 ppm), and diluent blank were made and analysed using the HPLC method described. The typical chromatograms for specificity were displayed in (Figure 3a to 3c).

**Table 1. Linear regression details for Gadobutrol and impurity-A, impurity-B and impurity-C**

| Parameter | Impurity-A | Impurity-B | Impurity-C | Gadobutrol |
|---|---|---|---|---|
| Linearity (ppm) | 3.2971–34.62 | 0.3788– 34.82 | 2.9757– 32.38 | 0.8314– 30.21 |
| Correlation coefficient (r) | 0.9988 | 0.9991 | 0.9968 | 0.9991 |
| Intercept value | 33855.835 | 154620.629 | -147462.62 | 454535.662 |
| Slope value | 41316.302 | 479640.138 | 189669.234 | -355480.676 |
| Square of Correlation coefficient ($r^2$) | 0.9976 | 0.9982 | 0.9937 | 0.9982 |
| 100% Y-intercept | 3.47 | 1.44 | -3.84 | -4.11 |

**Quantification and detection limits:** Quantification and detection limits were checked for impurities A, B and C at a concentration which gives an S/N fraction ≥10 and ≥3, respectively. The values of detection limit for Gadobutrol and impurities impurity-A, B and C were 0.2739 ppm, 1.0881 ppm, 0.1250 ppm and 0.9820 ppm, respectively. The quantification limits were 0.8314 ppm (Gadobutrol), 3.2971 ppm (impurity-A), 0.3788 ppm (impurity-B) and 2.9757 ppm (impurity-C). The quantification limit values for Gadobutrol and impurities A, B and C were confirmed by precision results. The determined %RSD of six area responses of Gadobutrol and impurity-A, B and C at their quantification limit level were 5.8% (Gadobutrol), 9.0% (impurity-A), 9.0% (impurity-B) and 11% (impurity-C).

**Linearity:** The linear quantity range for Gadobutrol and A, B and C impurities was checked in a quantity range from the quantification limit level (0.8314 ppm for Gadobutrol, 3.2971 ppm for impurity-A, 0.3788 ppm for impurity-B and 2.9757 ppm for impurity-C) to 150% of specification quantity limit (30.21 ppm for Gadobutrol, 34.62 ppm for impurity-A, 34.82 ppm for impurity-B and 32.38 ppm for impurity-C). A linear correlation was detected between area responses and concentrations of Gadobutrol and A, B, and C impurities in the range of quantities studied. The linear regression parameters were shown in Table 1.

**Method precision:** By analysing a Gadobutrol sample spiked with contaminants impurity-A (20 ppm), impurity-B

(20 ppm), and impurity-C (20 ppm), the method precision was confirmed (20 ppm). The method precision was determined by calculating the mean concentration and relative standard deviation of six different Gadobutrol, impurity-A, impurity-B, and impurity-C values (Table 2).

**Table 2. Precision study**

| Sample | Impurity: A (ppm) | Impurity: B (ppm) | Impurity: C (ppm) | Gadobutrol (ppm) |
|---|---|---|---|---|
| 1 | 21.00 | 21.50 | 21.30 | |
| 2 | 21.20 | 20.70 | 21.30 | |
| 3 | 21.20 | 20.20 | 20.80 | 9676 |
| 4 | 22.00 | 20.90 | 20.10 | |
| 5 | 21.50 | 21.40 | 21.30 | |
| 6 | 20.50 | 21.50 | 22.40 | |
| Average | 21.00 | 21.00 | 21.00 | NA |
| Standard deviation | 0.01 | 0.01 | 0.01 | NA |
| %RSD | 2.36 | 2.51 | 3.55 | NA |

**Table 3. Accuracy results**

| S.No. | Theoretical (%) | % Mean Recovery | | | |
|---|---|---|---|---|---|
| | | Impu: A | Impu: B | Impu: C | Gadobutrol |
| 1 | 50 | 106.7 | 103.0 | 106.9 | 108.7 |
| 2 | 50 | 103.4 | 102.0 | 102.0 | 105.8 |
| 3 | 50 | 102.5 | 104.0 | 106.9 | 96.2 |
| Average | | 104.2 | 103.0 | 105.3 | 103.6 |
| Standard deviation | | 2.2113 | 1.00 | 2.8290 | 6.5424 |
| RSD | | 2.1 | 1.00 | 2.7 | 6.3 |
| 1 | 100 | 108.4 | 109.6 | 104.9 | 93.8 |
| 2 | 100 | 108.4 | 107.6 | 104.9 | 86.1 |
| 3 | 100 | 110.0 | 105.6 | 102.5 | 89.9 |
| Average | | 108.9 | 107.6 | 104.1 | 89.9 |
| Standard deviation | | 0.9238 | 2.00 | 1.3856 | 3.8501 |
| RSD | | 0.8 | 1.9 | 1.3 | 4.3 |
| 1 | 150 | 98.3 | 112.5 | 105.6 | 90.4 |
| 2 | 150 | 100.3 | 114.5 | 98.7 | 91.1 |
| 3 | 150 | 97.8 | 113.5 | 101.6 | 90.4 |
| Average | | 98.8 | 113.5 | 102.0 | 90.6 |
| Standard deviation | | 1.3229 | 1.00 | 3.4646 | 0.4041 |
| RSD | | 1.3 | 0.9 | 3.4 | 0.4 |

**Accuracy:** Appropriate amounts of impurities A, B and C were spiked with Gadobutrol sample with replicates (n=3) at 50%, 100% and 150% of specification quantity limits. These samples were analysed by way of suggested HPLC method and determined the recovery of impurities A, B and C at each level (Table 3).

**Impurity-A, B and C stability in Gadobutrol solution:** Impurities-A, B and C spiked to Gadobutrol sample were stored at bench top and analysed by way of suggested HPLC method at 0 hr, 24 hr, 48 hr and 72 hr. Determined the recoveries of Gadobutrol, impurity-A, impurity-B and impurity-C. The recoveries of Gadobutrol, and A, B and C impurities obtained at 24 hr, 48 hr and 72 hr were compared with Gadobutrol and impurities recoveries at 0 hr (Table 4). The same set of experiments was repeated with impurities-A, B and C impurities spiked to Gadobutrol sample stored in refrigerator (Table 4).

**Forced degradation:** The chromatograms obtained from various stress tests like, involving acid, base, peroxide, humidity and thermal degradation on Gadobutrol are shown in (Figure 4a-4e). (Table 5) summarizes the forced degradation findings. Blank peak, on the other hand, did not overlap impurity A, B, or C peaks (Figure 3a). The retention periods for Gadobutrol (Figure 3b), impurities A, B, and C (Figure 3c) are all different, according to chromatograms. This shows that DFP had no effect on impurity-A, impurity-B, or impurity-C analyses. As a result, it's a very selective method (Marcello et al. 2012; Ravisankar et al. 2015; Ahmad et al. 2022; Lalic et al. 2022). The determined %RSD of 6 area responses of Gadobutrol

and impurities A, B and C at their quantification limit levels confirmed sensitivity and precision at quantification limit concentrations (Marcello et al. 2012; Ravisankar et al. 2015; Leistner and Holzgrabe 2021).

**Table 4. Consequences of Impurities-A, B and C stability in Gadobutrol solution**

| Component | ppm determined | | % Difference | ppm determined | % Difference | ppm determined | % Difference |
|---|---|---|---|---|---|---|---|
| | Initial study | After 24 hr | | After 48 hr | | After 72 hr | |
| **Stored at bench top** | | | | | | | |
| Impurity-A | 26.00 | 26.60 | 0.001 | 25.70 | 0.003 | 26.40 | 0.02 |
| Impurity-B | 20.70 | 20.90 | 0.02 | 22.90 | 0.022 | 24.60 | 0.039 |
| Impurity-C | 21.30 | 19.80 | 0.015 | 20.30 | 0.01 | 21.60 | 0.003 |
| Gadobutrol | NA | 102.5 | NA | 105.5 | 3.00 | 104.2 | 1.7 |
| **Stored in refrigerator** | | | | | | | |
| Impurity-A | 26.00 | 26.70 | 0.007 | 25.70 | -0.003 | 25.90 | -0.001 |
| Impurity-B | 20.70 | 20.90 | 0.002 | 23.00 | 0.023 | 24.30 | 0.036 |
| Impurity-C | 21.30 | 21.30 | 0.00 | 21.10 | -0.002 | 20.50 | -0.008 |
| Gadobutrol | NA | 99.4 | NA | 95.8 | -3.6 | 102.8 | 3.4 |

NA – not available

**Figure 4a: Chromatogram of acid stressed Gadobutrol sample**

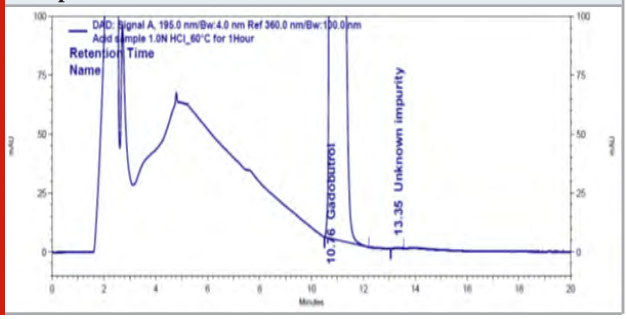



**Figure 4b: Chromatogram of base stressed Gadobutrol sample**

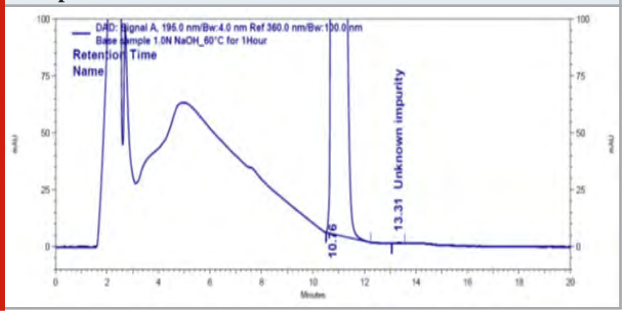



**Figure 4c: Chromatogram of oxidative stressed Gadobutrol sample**

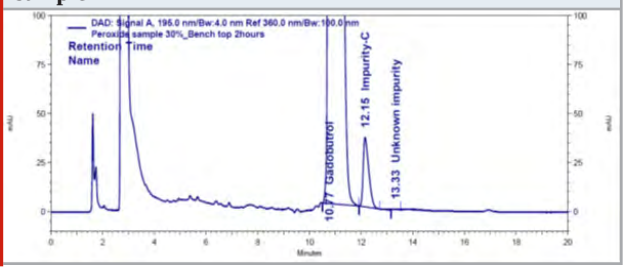



**Figure 4d: Chromatogram of dry heat stressed Gadobutrol sample**

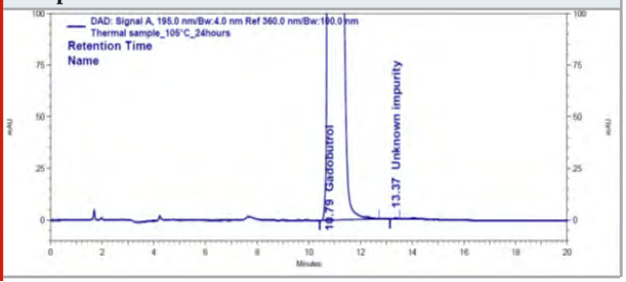
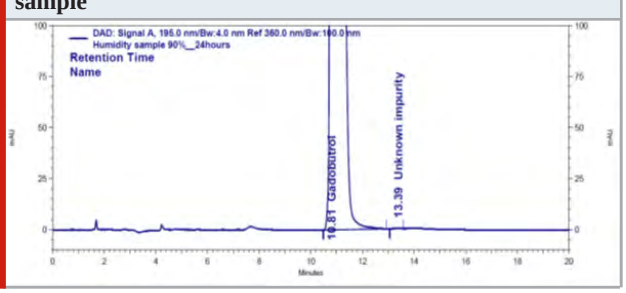



**Figure 4e: Chromatogram of humidity stressed Gadobutrol sample**




The coefficient of correlation (Table 1) values for Gadobutrol and impurities A, B and C disclose the process was of linear inside the concentration range determined (Soumia et al. 2017; Kowalska et al. 2022). The relative standard deviation (Table 2) determined for impurities-A, B and C was noticed as 2.36% to 3.55% which evidenced that the protocol was precise for evaluation of present impurities (Betz et al. 2011; Musmade et al. 2021). The evaluated values of recoveries (Table 3) for impurities A, B

and C for the suggested method proved that the method was accurate enough for the evaluation of the present impurities in the Gadobutrol sample (Betz et al. 2011; Musmade et al. 2021).

In the chromatograms (Figures 4a-4e) of all Gadobutrol degraded samples, all degradation peaks were resolved well from Gadobutrol peak, and did not display any interfering at the retention times of Gadobutrol and impurities A, B and C. Gadobutrol was realised to be more stable in acid, alkali, thermal and humidity stress conditions but sensitive to peroxide made oxidative condition. These findings for the suggested method proved that method was stability indicating enough for evaluation of the studied impurities in Gadobutrol sample (Ganpisetti et al. 2020; Manchuru et al. 2020; Karaer et al. 2022).

**Table 5. Forced degradation results of Gadobutrol sample**

| Degradation condition | Gadobutrol degraded % | % Occurrence of | | | | |
|---|---|---|---|---|---|---|
| | | Impurity: A | Impurity: B | Impurity: C | Unknown impurity | Total Impurities |
| Control sample | 0.02 | N.D. | N.D. | N.D. | 0.02 | 0.02 |
| Acid: 1.0 N HCl@60°C/1hr | 0.02 | N.D. | N.D. | N.D. | 0.02 | 0.02 |
| Base: 1.0 N NaOH@60°C/1hr | 0.02 | N.D. | N.D. | N.D. | 0.02 | 0.02 |
| Peroxide: 30% $H_2O_2$@ bench Top/4 hr | 2.11 | N.D. | N.D. | 2.09 | 0.02 | 2.11 |
| Thermal: 105°C/24 hr | 0.02 | N.D. | N.D. | N.D. | 0.02 | 0.02 |
| Humidity: 90% RH/24 hr | 0.02 | N.D. | N.D. | N.D. | 0.02 | 0.02 |

N.D. – not detected

## CONCLUSION

The findings of the present study shows that HPLC-dependent method is a reliable and effective to detect and evaluate Gadobutrol and impurities A, B and C simultaneously in the Gadobutrol sample was developed and validated. The parameters validated (linear regression, accuracy, robustness, precision, specificity, detection limit, devise suitability, quantification limit) for Gadobutrol and impurities A, B and C were in line with ICH criteria requirement. Our results indicated that Gadobutrol sample quality can be assessed using the method proposed in this study.

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**Biotechnological Communication**

# Effect of Nitrogen on the Biomass Production and Lipid Accumulation of *Micractinium reisseri*: A potential Microalgal Strain for Bio-fuel Production

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## ABSTRACT

The potential microalgal strains that are robust and display high growth and lipid accumulation rates are an important prerequisite for using them as a bioenergy source. We have isolated and screened six morphologically different microalgae strains, isolated from the Sabarmati River in Gujarat, India. The growth rates and lipid productivity of all six microalgae were assessed. Three potential microalgae strains were screened based on maximum biomass and lipid production. We named them MA001, MA002 and MA003. We identified MA001 as *Micractinium reisseri* based on its 28S rRNA sequencing. The *M. reisseri* showed an optimal growth rate of 2 g/L (dry weight) and 52 % lipid content after 20 days of cultivation in a normal artificial saline medium. Further analysis of lipid accumulation in *M. reisseri* was investigated at different concentrations of nitrogen. *M. reisseri* accumulated the highest amount of lipid under nitrogen starvation.

**KEY WORDS:** ARTIFICIAL SALINE MEDIUM, BIODIESEL, BIOENERGY, *MICRACTINIUM REISSERI*, MICROALGAE.

## INTRODUCTION

Biofuel has caught substantial attention worldwide now-a-days as an alternative fuel due to its capability to adapt with gasoline for a maximum 85% blend without any engine modification. Subsequently, the suitability of microalgae for biofuel is being continuously queried by researchers and environmentalists (Hossain et al. 2017; Iqbal and Khan 2018; Hossain et al. 2019; Taofeeq et al. 2021). A well-managed and equipped microalgal bio-refinery can produce biodiesel and other value-added products such as protein, carbohydrates and a range of fatty acids (FAs) e.g., omega-3 fatty acids etc., and some non-nutritional value-added by-products such as bioplastic etc. (Beckstrom et al. 2020; Rajesh et al. 2020). However, the commercial production of microalgae biofuel is unfortunately offset (Chozhavendhan et al. 2022).

The oil content of the microalgae, the nitrogen content of the wastes, and the extraction and transesterification efficiencies significantly affect the biodiesel production in the integrated process, implies that improving these parameters is significant in increasing the feasibility of the integrated process (Deribew and Abubeker 2020; Zewdie and Ali 2020; Chozhavendhan et al. 2022). It was estimated that the current cost of producing 1 ton of microalgal biomass with an average 55% (w/w dry weight) oil content needs to be reduced by 10-fold in order to be competitive with petroleum diesel (Nazifa et al. 2021; Chandima and Judy 2022).

Therefore, photobioreactors and other effective methods have been designed for cultivation to enhance the rate of production of biomass (Patel et al. 2019; Zhang et al. 2022). However, one more factor, i.e., the potential of microalgae, cannot be ignored while improvising the production strategies. The high-yielding microalgae strain can directly translate into an overall increase in biodiesel production. Also, the lipid production during normal growth needs to be distinguished from lipid accumulation in response to adverse conditions e.g., nutrient starvation (Asma et al. 2022). The quantitative and qualitative differences in the lipid content of a given species affect the quality of biodiesel and its ability to meet fuel standards (Knothe 2005; Hu et al. 2008; Asma et al. 2022).

To date, research efforts have been focused on microalgal lipid production under several different growth conditions (Wagenen et al. 2012; Shafik et al. 2015, Reeza and Mallick 2021; Zhang et al. 2022). The direct comparison of various

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microalgae in different studies is unreliable because various researchers have used different growth conditions and experimental parameters for each species and also different methods have been used for lipid extraction (Zhang et al. 2022). However, the one that has a large accumulation of lipids proves to be a promising species for biodiesel production at a commercial level. Therefore, it is very important that one should have a potential microalgae strain along with a potential system of biodiesel production (Zhang et al. 2022).

In this study, few microalgae strains were isolated from Sabarmati River, Ahmedabad, Gujarat, India to screen the potential candidate for biomass and biofuel production. Total six microalgae strains were screened, of which three of them were shown to produce higher biomass and total lipid content. We named these three potential microalgae strains as MA001, MA002 & MA003. After various nutrient media screening, the artificial saline medium (ASM) was proved to be the most suitable medium for the cultivation of MA001, MA002 & MA003. After 20 days of incubation MA001, MA002 & MA003 were assayed for the production of total biomass estimated as 0.2%, 0.1% and 0.04% (dry weight) and total lipid content was 52%, 47% and 65% (w/v) respectively. Further molecular identification by using 28S rRNA sequencing, identified the strain MA001 as Micractinium reisseri. Since, *M. reisseri* was found to produce higher biomass among all three screened potential strains; therefore, further studies were done to analyze the characteristics of *M. reisseri*. In this study the importance of nitrogen for the biomass production and lipid accumulation in *M. reisseri* are discussed.

## MATERIAL AND METHODS

A total of six water samples (~2 L each) were collected from Vasna Barrage, Sabarmati Riverfront, Ahmedabad (Latitude 23.0080261 & Longitude 72.5716496) from 10 and 20 feet away from the river bank at three surfaces viz. surface water, 1 foot and 5 feet below the water surface. The temperature and pH of the collected water samples were noted on the spot at the time of collection. Isolation and purification of microalgal strains was done by serial dilution technique followed by plating of each dilution sample on four nutrient media viz., C10, B11, artificial saline medium (ASM) and artificial saline water (ASW) with 1% agar, pH 8.0 and were allowed to incubate under controlled environmental conditions in the growth room where the temperature was set at 25 $^{o}$C, light intensity of 3000 Lux, white LED light with 16 hours light and 8 hours dark photoperiod and 80% relative humidity (RH). A total of around 100 microalgal colonies along with the other microbial colonies were obtained. After several steps of isolation and purification, there were six green microalgal colonies with different morphology and green shades were selected.

Out of six selected colonies, one was successfully grown in B11 medium, the second was in C10 medium, the third was in ASW medium and the remaining three were grown in ASM medium. All six strains were shown to be axenic microalgae cultures. For further studies, these six axenic microalgae cultures were analyzed for their characteristics.

Based on the estimated rate of growth and total oil content in all six selected microalgae strains, the three microalgae strains were screened for the most potential microalgal candidates and, therefore, selected for further studies. These three microalgae strains were labelled as MA001, MA002 & MA003 for which ASM medium was the most supportive nutrient medium. A morphological study of the selected microalgae strains was done under the fluorescence microscope (Nikon) at 40x and 100x magnification. Growth parameters of the microalgae strains were measured every seven days of incubation up to 70 days. The cell density was measured using a hemocytometer. The microalgae biomass in a 10 ml liquid culture was measured by centrifugation at 4000 rpm for 30 min. The whole set of experiments was done in triplicate. The data obtained at different incubation periods was recorded.

For the molecular identification of microalgae strain 001, the fresh sample was harvested in 10 mL of liquid culture by centrifugation. Microalgae sample was washed with sterile Millipore water and dried at room temperature (~30°C) for a few hours to remove excess liquid. The dried microalgae MA001 sample was subsequently for DNA extraction. The air-dried microalgae cells (1 g) were ground to a powder in a pre-chilled mortar and pestle. The powder was then transferred into a 2 µl microcentrifuge tubes. Then 500 µl of the extraction buffer (CTAB-3% (w/v); Tris HCl-100mM; NaCl-2M; EDTA-25mM; Mercaptoethanol-4% (v/v); PVP-5% (w/v); pH 8.0) was added into it and mixed well. The mixture was incubated for 30 minutes at 70°C with periodic shaking. After incubation, the mixture was cooled at room temperature and then 500 µl chloroform: isoamyl alcohol (24:1) was added and mixed gently for 15 min. The mixture was centrifuged at 12000 rpm for 15 min. The above aqueous phase (about 500 µl) was transferred to a new tube and then added ice-cold isopropanol (700 ml).

The whole content was mixed gently and kept at -20 $^{o}$C for 10 min. Then, centrifuged the tubes at 10,000 rpm for 5 min. The supernatant was discarded. Cold 80% ethanol was added to the pellet to wash it twice by centrifugation at 10,000 rpm for 5 min. The ethanol was discarded, and the pellet was air dried. The pellet was re-suspended in 50 µl 1X TE buffer (10mM Tris HCl; 1Mm EDTA; pH 8.0) and stored at -20 $^{o}$C. DNA concentration and purity were determined by measuring the optical density using a UV- spectrophotometer (SHIMATZU). For this, 10 µl of DNA sample was diluted with 100 µl of 1X TE buffer (10 X dilution), and the optical density was measured at 260 nm in a UV-Spectrophotometer (SHIMADZU) against 1X TE buffer. The DNA concentration was then calculated according to the standard method. Optical density (OD) was also taken at 280 nm (corresponding to protein), 230 nm (corresponding to RNA) and 320 nm (contamination). Total DNA purity was tested by a ratio of OD values at 230:260:280. Species identification of the microalgae strain MA001 was done by the molecular sequencing of the 28S rRNA subunit.

The primer sequence used for the PCR reaction was: F- "ACCCGCTGAATTTAAGCATA" and R- "CCTTGGTCCGTGTTTCAAGA"

The produced DNA sequence was analyzed using BLAST to confirm the sequence identification.

Four sets of ASM media supplemented with three different concentrations of nitrogen compound were used to cultivate the microalgae strain MA001. ASM medium without nitrogen supplement was used as a control. In ASM medium, three different concentrations of sodium nitrate ($NaNO_3$) supplement were used: 1.25, 2.5, and 5.0 g/L. The microalgae strain, MA001 with a cell density of 6.58 x $10^5$ cells/ml was inoculated in all four sets of ASM medium containing 0, 1.25, 2.5 and 5.0 g/L concentrations of NaNO3. The microalgae inoculum and ASM medium ratio were maintained at 1:100 taken in 250 ml Erlenmeyer flasks. All inoculated flasks were incubated in static condition in a controlled environment at a temperature 25 $^oC$, a light intensity of 3000 Lux, white LED light with a photoperiod 16 hours of light and 8 hours of darkness, and a relative humidity (RH) of 70-80 %.

Growth parameters and biochemical assays of the growing microalgae strain were done on the 5$^{th}$, 10$^{th}$, 25$^{th}$ and 40$^{th}$ days of incubation. A 10 ml liquid microalgal culture was harvested from each flask and used to study the growth parameters such as (i) cell density; (ii) wet biomass; (iii) dry biomass; (iv) chlorophyll content; (v) total lipids and (vi) neutral lipids by using standard methods. Cell density in 1 ml of algal suspension culture was measured using a hemocytometer and optical density ($OD_{650}$) was taken using an Ultraviolet-Visible spectrophotometer (SHIMADZU). All measurement were done in triplicates by taking algal culture from three parallel flasks of the same test sample. The linear correlation between the cell density of *M. reisseri* and $OD_{650}$ was established for the subsequent *M. reisseri* density determination using the equation given by Li et al. (2010). The software EXCEL was applied to process the data, draw the scatter plot and calculate the correlation coefficient.

The linear correlation between $OD_{650}$ and cell density was determined by the equation: D (x$10^6$ cells/ml) = 79.311x+70957; ($R^2$ = 0.997). Wet biomass was determined by the centrifugation method. First weighed the empty centrifuge tube (15 ml volume). 10 ml of microalgae culture was taken in each centrifuge tube and centrifuged at 4000 rpm for 30 min. After centrifugation, the supernatant was decanted and again weighed the centrifuge tube with the microalgae pellet. The weight difference of centrifuge tubes before and after centrifugation was recorded. The centrifuge tubes with wet microalgae pellets were allowed to air-dry for 24 hours. The centrifuge tubes with dry microalgae pellets were weighed again. The weight difference between empty centrifuge tubes and the same centrifuge tube with an air-dried microalgae pellet after centrifugation was recorded.

For chlorophyll estimation the microalgae cells were harvested from a 10 ml suspension culture and washed with sterile Millipore water. Microalgal cells were partially dried at room temperature and then 10 ml of methanol was added to them. The cells were mixed thoroughly with the help of a glass rod, and then the content was transferred to an Erlenmeyer flask. The flask was closed tightly with aluminum foil and kept in a water bath at 60oC for 30 min. Then, it was allowed to cool at room temperature. Methanol content was decanted in a 15 ml centrifuge tube. The final volume was adjusted to 10 ml by adding methanol. The tube was centrifuged at 8000 rpm for 10 min at ambient temperature. The supernatant was separated and the optical density (OD) of the supernatant was measured at the wavelengths of 663 nm and 645 nm in the UV-Spectrophotometer (SHIMADZU). Methanol was used as a control. The obtained values were used in the formulas, described by Gu et al. (2016) for the estimation of photosynthetic pigments.

Chlorophyll a (mg/g) = (12.7 x $OD_{663}$) − (2.59 x $OD_{645}$);
Chlorophyll b (mg/g) = (22.9 x $OD_{645}$) − (4.7 x $OD_{663}$);
Chlorophyll total (mg/g) = (8.2 x $OD_{663}$) + (20.2 x $OD_{645}$).

The Nile red staining was used to determine the lipid globular structures inside the microalgae cells as described by researchers (Matsunaga et al. 2009; Elumalai et al. 2011). The cells of microalgae strain MA001 were isolated from 1 ml suspension cultures in ASM media on the 40th day of incubation. The culture was centrifuged at 1,500 rpm for 10 min and sediment was washed with saline solution (0.5 ml) 2-3 times. The collected microalgal cells were suspended in 0.5 ml of Nile red solution (0.1 mg of Nile red/ml of acetone) and incubated for 10 min at room temperature. The stained microalgal cells were washed with sterile reverse osmosis (RO) water, and the intracellular lipid content was observed under a fluorescent microscope (NIKON ECLIPSE E200). The neutral lipid content of MA001 at three different concentrations of nitrogen and control samples were determined by modified fluorescence protocol (Chen et al. 2009). 1 ml of microalgae suspension culture was taken in a microcentrifuge tube. Similarly, 1ml of sterile reverse osmosis (RO) water was taken in another microcentrifuge tube that was used as a blank. 50 µl DMSO (dimethyl sulfoxide) was added to all the tubes and mixed well. The tubes were kept in a water bath at 40$^oC$ for 1 min.

Tubes were cooled at room temperature and 10 µl Nile red stock (125 µg of Nile red/ml in acetone) was added. After a thorough mixing, the tubes were again kept in the water bath at 40$^oC$ for 30 min. with an intermediate shake. After algal cells were stained, fluorescence emissions were recorded with the Elisa Reader technique equipped with a 96-well plate reading mode. Analysis for all the samples was performed in triplicates. Fluorescence emissions were recorded for the stained microalgal cells at excitation and emission wavelengths of 490 nm and 580 nm, respectively, by using an ELISA Reader (Thermo Scientific). Observations were made at 10, 25 and 40 days of incubation. Total lipid was measured by the method Bigogno, et al. (2002). 100 mg of dry microalgae biomass was taken in a glass vial and 5 ml solvent I (10% DMSO in methanol) was added into it. The vial caps were tightly closed and placed in a 45$^oC$ water bath with occasional shaking for 45 minutes.

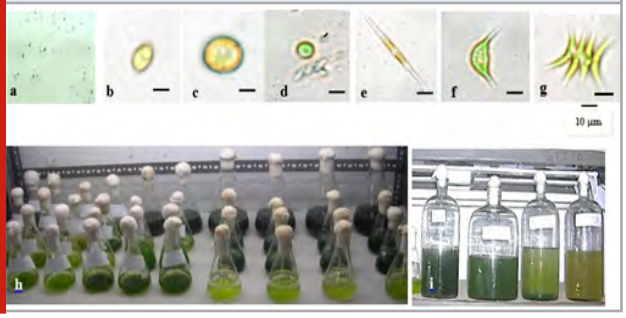
The vials were then cooled down to room temperature. 5 ml of solvent II (diethyl ether: hexane (1:1)) was added to it and it was placed in a 45$^oC$ water bath for 60 minutes. After cooling at room temperature, the content was transferred into 15 ml

centrifuge tubes. Tubes were centrifuged at 5000 rpm for 10 min. The supernatant was transferred into a separating funnel. 10 ml of sterile distilled water was added to it. The content was mixed thoroughly and allowed to settle down for one night. Two solvent phases were separated. The upper organic phase, consisting mainly of lipids was collected in a pre-weighed vial. Then weighed the organic lipid content obtained from 100 mg of dry microalgae biomass.

## RESULTS AND DISCUSSION

Light to dark green colonies were formed after about 10-20 days of incubation (Figure 1a). Six randomly selected colonies were observed under the light microscope. All colonies were shown to have common characteristics of the family Chlorophyceae. The size of the individual cells of each strain was in the range of 8-10 µm. All six colonies were shown to have different shades of light to dark green, which can be seen with the naked eye. The shape of all six types of cells varies from oval to spherical to spindle; each cell with a single nucleus as shown in figure 1(b-g). Strains MA001, MA002 and MA003 were grown successfully in ASM medium for 70 days in 100 mL Erlenmeyer flasks (Figure 1h). They were further cultivated in big glass jars with a 5 liters capacity in the ASM medium (Figure 1i). There was no significant difference in the growth patterns that were observed even with an increase in the volume of ASM medium from 250 ml to 2500 ml. The ratio of inoculum to nutrient media was fixed at 1:100. The morphological characteristics of MA001, MA002 and MA003 are shown in table 1. After 20 days of incubation of microalgae strains MA001, MA002 & MA003, the estimated recovery of biomass was 2 g/L, 1 g/L and 0.4 g/L (dry weight) and lipid content was 52%, 47% and 65% respectively. The results indicated that biomass production was highest in the MA001 strain and lipid production was highest in the MA003 strain among all three.

**Figure 1: (a) Microalgae colonies on media plates; (b-g) Light microscopic images of six isolated microalgal strains; (h) axenic microalgae cultivation in flasks and (i) jars.**




Molecular identification was done for the strain MA001. Genomic DNA of MA001 was sequenced for the 28s RNA subunit from outsourcing (Eurofins). The nucleotide sequence of a small DNA fragment (~400 bp) is shown in figure 2. NCBI-Blast search of the sequence of a small DNA fragment of MA001 showed 100% similarity with microalgae - *Micractinium reisseri* belong to the family Chlorophyceae.

The effect of nitrogen on the growth and development of *M. reisseri* was studied under controlled environmental conditions. In general, the ASM medium contains 2.5 g/l $NaNO_3$ as a nitrogen supplement, which we considered a normal concentration (1N2). The remaining two concentrations were half (1/2N2) and double (2N2) the normal nitrogen supplement concentration. ASM media without any nitrogen supplement was used as a control (-N2). *M. reisseri* was harvested on the 5, 10, 25 and 40 days of incubation. The growth parameters of *M. reisseri* were determined after each harvest. Cell density or the biomass productivity of *M. reisseri* was increased from low to high concentrations of nitrogen. Cell growth was also observed in control.

Figure 3 shows the total cell biomass with respect to the linear growth curve of M. reisseri with increasing concentrations of nitrogen. Observations were made for 40 days of incubation. It was observed that without nitrogen compound, there was no significant increase in the cell density. However, with an increase in nitrogen concentration in the ASM medium, there was a significant increase in the cell density with an increase in incubation period. A drastic increase in cell density was observed on the 40$^{th}$ day of incubation when there was the highest concentration of nitrogen (5 g/L). The growth rate and cell density of *M. reisseri* grown on ASM medium with and without nitrogen supplement demonstrated that biologically available nitrogen support enhances $CO_2$ fixation for photosynthesis, which brings about the cell growth. However, with a nitrogen supplement, the cell growth is further enhanced. The highest level of chlorophyll-a in 2N2 concentration was observed, followed by a decreasing order of chlorophyll-a content with decreasing concentrations of nitrogen, viz., 1N2, 1/2N2, 0N2 was observed up to the 40th day of incubation (Figure 4). However, the estimated amount of chlorophyll a in 1N2 was very close to that in 2N2. The medium without nitrogen supplement (-N2) contained the least chlorophyll-a of all (Reeza and Mallick 2021; Zhang et al. 2022).

There was a marginal variation in the level of chlorophyll-b content in *M. reisseri* when grown at four different concentrations of nitrogen up to 25 days after inoculation (Figure 5). However, on the 40th day of incubation, there was a sudden increase in the level of chlorophyll b observed in all three concentrations of nitrogen. The highest amount of chlorophyll-b was observed in 2N2 followed by less in 1N2 and least in 1/2N2. *M. reisseri* growing in ASM medium without any nitrogen supplement (–N2) determined a negligible amount of chlorophyll-b. In all concentrations of nitrogen there was a slight decrease in the total chlorophyll up to 25 days, but it suddenly increased on the 40$^{th}$ day of incubation. (Figure 6).

It has been observed that when the nitrogen supplement was absent or of less amount in the ASM medium, there was the least production of total chlorophyll in the *M. reisseri*. Photochemical measurements show that nitrogen supplementation does not inhibit photosynthesis in phytoplankton. However, the rate of Photosystem-II re-oxidation becomes faster and photosynthetic electron transport is enhanced during growth on a high level of $NaNO_3$ and cells maintain overall photosynthetic efficiency. As a result, the higher the level of chlorophyll, the greater the growth and development of microalgal cells. The selection of solvent is very important in the pigment extraction process because the polarity of the solvent used for the extraction process plays a major role. In the present work, the pigment

extraction with methanol was nearly completed in *M. reisseri* cells. Many factors, such as the cell wall structures of M. reisseri, a well performed homogenization and a complete extraction, facilitated the isolation of the pigments. It was suggested in earlier studies that the chlorophyll-a level was found to be the same in all microalgae groups (Chiu et al. 2009; Nazifa et al. 2021).

**Table 1. Characteristics of 20 days old microalgae strains**

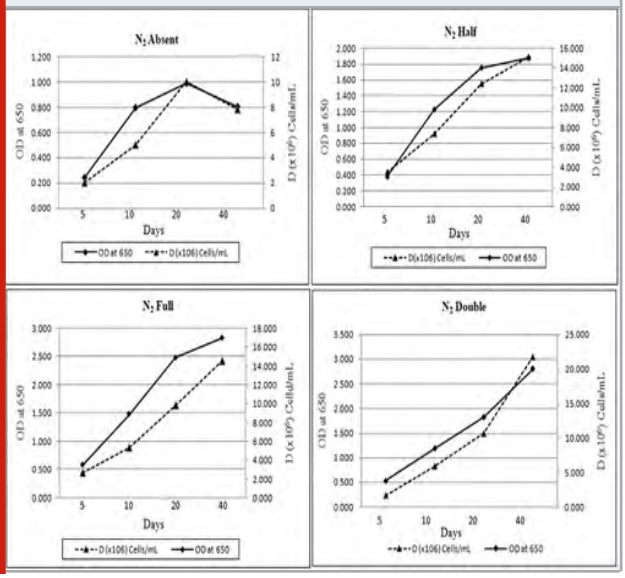
| Parameter | MA001 | MA002 | MA003 |
|---|---|---|---|
| Cell Morphology | Dark green, oval. and round, small, always dispersed | Light green, round, bigger than MA001, sometimes in group or dispersed. | Light green, sickle shaped, pointed ends, flexible, always dispersed. |
| Dry Biomass ( g/L) | 2 | 1 | 0.4 |
| Lipid content (%) | 52 | 47 | 65 |
| De-oiled cake (g) | 1.2 | 1.1 | 0.45 |

**Figure 2: 28S rRNA sequence of MA001 DNA fragment.**

DNA Sequence:

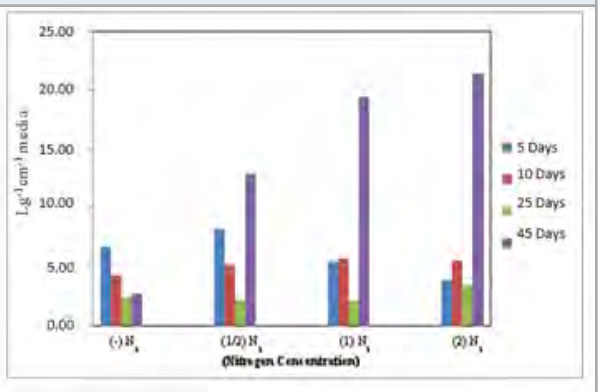
GCGAACCGGGCAAAGCCCAACTTGAAAATCTCCACCCTCCGGCTGGCGAATTGTAGTCTAG
AGAAGCGCTCTCTGCCCCAGTCTGGTCCGAAGTCCCCTGGAAAGGGCGCTCAAAGAGGGTG
AGAACCCCGTTGGGACCGGAGCCTGGGGCTCCACGGGACGCTTTCGAAGAGTCGGCTTGTTT
GGGAATGCAGCCCAAAGCAGGTGGTAAATCCCATCTAAGGCTAAATACCGACGGGAGACCG
ATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGCAACTTTGAAAAGAGAGTTAAAAAGT
ACTTGAAATTGTTGAGAGGGAAGCGATTGGATCCCACGGGTGCGCCCAGGCACACGCCTGC
TTCACGGTTGGTTGAATGCGCTGGGCGCTGGTCAGCATGGGTTGTGCGGGCGGGATAAAACC
GGGGGTTGTTACCCCGGCTATGCCCCCCGGTCGACCGAGGTACGAAGGGCGCTCTTTGAGTC
CTCGGGGAAC

**Figure 3: Cell Density of *M. reisseri* cultivated in ASM medium with $N_2$ absent, 1.25 g/L ($N_2$ half), 2,5 g/L ($N_2$ full) and 5.0 g/L ($N_2$ double) concentrations.**




However, we have found that the level of chlorophyll a in *M. reisseri* varies at different concentrations of nitrogen. With an increase in nitrogen, the level of chlorophyll-a was increased due to the increase in cell growth. In other words, the amount of chlorophyll a pigment was directly proportional to the cell biomass at a specific nitrogen concentration. In a previous study, it was reported that the level of chlorophyll is found higher in the microalgae present in fresh water and in an environment where much light and temperature stratification are not seen (Li et al. 2010). It was also determined that there were changes at the pigment level of algae species that live in an environment where light stratification is seen (Daphne et al. 2021). That is why one of the opinions is that the reason for the chlorophyll-a level to be higher with an increase in time of incubation and nitrogen concentration is linked with light and temperature stratification during the course of time when the samples were taken. Li et al. (2008) reported that the accumulation of $CaCO_3$ was found to be very high on the cell walls of almost all microalgae, e.g., *Codium tomentosum* and *Cladostephus verticillatus*, which hinders the extraction process (Chandima and Judy 2022).

**Figure 4: Chlorophyll-a estimated in *M. reisseri* grown at four different concentrations of nitrogen in ASM media.**




In our experiment, cell homogenization was a difficult step that might be due to $CaCO_3$ accumulation, and therefore, all pigments were not extracted completely. As a result, chlorophyll-b was determined to be less in concentration as compared to chlorophyll-a in *M. reisseri*. An optimal protocol for ELISA was successfully applied to *M. reisseri* for lipid determination. We have used Nile red and DMSO (dimethyl sulfoxide) solutions to improve the effectiveness and efficiency of lipid fluorescence. The microalgal extracts contained pigments that quenched Nile red fluorescence. Therefore, DMSO acts as a mild bleach solution to destroy pigments. DMSO was also introduced to microalgal samples as

the stain carrier at an elevated temperature (Chen et al. 2009). After the algal cells were stained with Nile red and DMSO, the fluorescence was measured on a 96-well-microplate reader (ELISA) using medium scan control and high PMT detector voltage mode at the excitation and emission of 490 nm and 580 nm.

**Figure 5: Chlorophyll-b estimated in *M. reisseri* grown at four different concentrations of nitrogen in ASM media.**

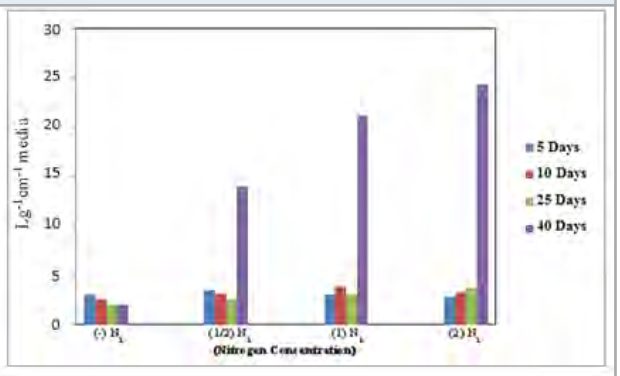



**Figure 6: Total chlorophyll estimated in *M reisseri* grown at four different concentrations of nitrogen in ASM media.**

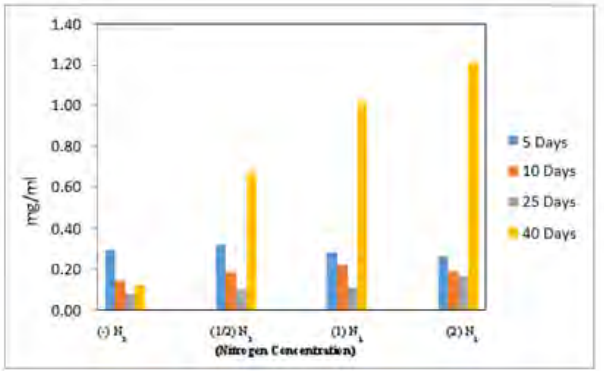
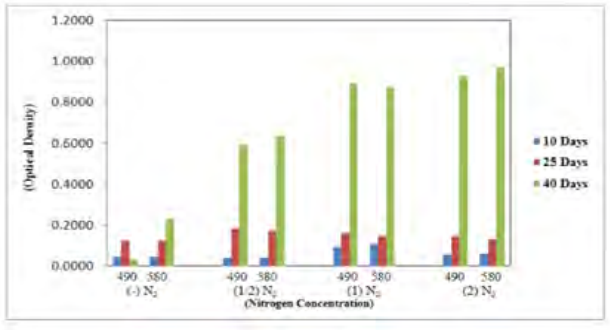



**Figure 7: Total lipids in *M. reisseri* grown at four different concentrations of nitrogen in ASM media (Optical density was measured at 490 and 580 nm).**




All *M. reisseri* cells contain measurable amounts of neutral lipids in the normal growth state. The observed fluorescence is not the result of inherent cellular fluorescence from chlorophyll as we have measured untreated *M. reisseri* suspension cultures without Nile red dye (control) as well as the ASM medium containing Nile red alone as an auto florescence. The amount of florescence is considered as an indication of the lipids. In table 1, we have mentioned that the total lipid content (polar and non-polar) in *M. reisseri* is 52 % of the total cell biomass (dry weight). In the ELISA reader, the lipid globular molecules were reacted with Nile red and gave a yellow florescence. Figure 8 shows the increase in optical density of *M. reisseri* suspension at 490 and 580 nm with an increase in nitrogen concentration of ASM medium. This is because the increasing nitrogen concentration in the nutrient medium would decrease the lipid accumulation in the microalgal cells. Due to the least availability of lipid molecules, more of the Nile red dye would retain its red colour and consequently increase the optical density with respect to control.

Nile red (9-(Diethylamino)-5H benzo [α] phenoxazin-5-one) staining is specifically used to identify the intracellular lipid droplets from the biological samples (Greenspan et al. 1985). The results indicated that *M. reisseri* could not be affected by Nile red staining since oil droplets were not clear and the whole cells were stained red. Similar results were reported earlier in a few other green isolates. Sayeda et al. (2013) reported that certain blue green isolates were affected by the dye, including *Microcystis aeruginosa* and *Phormidium rimosum* where the yellow stained parts were clear. However, Chroococcus turgidus, *Oscillatoria limnetica* and *Oscillatoria limosa* cells showed yellow florescent colour under a florescent microscope even without adding the dye. So, this gives false results when referring to lipid content.

The florescent method has been successfully applied to the determination of lipids in certain microalgae, but has been unsuccessful in many others, particularly those with thick, rigid cell walls that prevent the penetration of the dye (Held 2011). Judith et al. (2015) also mentioned that many of the microalgae strains do not give yellow colour with Nile red for lipid test. Indeed, interactions of the dye with proteins and/or other cellular components in cytosolic cells are major delay factors for colour development. He explained that fluorescence and staining kinetics depend on the microalgae species and the size of lipid droplets as well as on the amount of the latter. Li et al. (2008) reported that increasing the stress in the growth conditions can increase lipid accumulation in microalgal cells. Nitrogen stress is one of them. There are reports that confirm the higher proportions of lipids (up to 60%) in microalgae obtained under nitrogen starvation (Bigogno 2000; Christos et al. 2020).

The fatty acid composition and fatty acid content changed with the ageing of the culture. Consequently, the total lipid content increased dramatically with an increase in the age of microalgae, at least up to 30 to 40 days. However, the non-polar or neutral lipid accumulation is an important aspect in terms of its biofuel production. Certain microalgal species can be induced to synthesize and accumulate extremely high proportions of triacyleglycerate (TAG). Stewart and Coyne (2011) describe chimeric enzymes that are involved in maintaining cell growth in the presence of nitrogen.

## CONCLUSION

The findings of the present study has presented the potential to produce biodiesel from the microalgae *M. reisseri* - a model for neutral lipid production. The strength of *M. reisseri* is to predict with various morphological characteristics, developed at high nitrogen limitation to starvation. Both chlorophyll a and b were present in *M. reisseri*. The compositional information and methodology presented in this paper are valuable references for future studies on microalgae strain selection and algae cultivation, optimization, as well as for the production of biodiesel from microalgae.

## ACKNOWLEDGEMENTS

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**Data Availability Statement:** All data / results / information is available with the authors and can be shared on a reasonable request made to the corresponding author when required.

**Conflict of Interests:** Authors declare no conflict of interests to disclose.

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**Pharmaceutical Communication**

# Potential Impact of *Azadirachta indica* and Syzygium aromaticum on Growth and Development of Significant Cultivable Oral Bacterial Flora

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## ABSTRACT

Resistance has been reducing the effectiveness of antibiotics for the past few decades. Researchers are constantly investigating new herbal medicines which can be a better option as well. Aqueous and ethanol extraction method were followed to obtain clove buds and neem twig (datum) extracts. The obtained extracts were tested against selected bacteria using the well diffusion method and broth dilution method to assess the antimicrobial activities. The obtained data were recorded as MIC50 MIC80 and MBC (minimum bactericidal concentration). The tremendous antibacterial activities in extract of *Azadirachta indica* and *Syzygium aromaticum* were observed highest in 6.25 mg/mL of ethanol extraction method. It is concluded that neem and clove showed tremendous antimicrobial activities and both have been found quite effective in oral health even today.

**KEY WORDS:** ANTAGONISTIC ACTIVITY, CARIOGENIC BACTERIA, HERBAL EXTRACT, MBC AND MIC50.

## INTRODUCTION

Oral bacterial flora is found as both good and bad bacteria. Some behaves as commensals as they help maintain oral eco balance such as *Lactobacillus acidophilus*. Although they are also cariogenic bacteria. In addition, some behaves as opportunistic pathogens like *Streptococcus mutans* which is the most cariogenic bacteria. In fact, opportunistic pathogenic bacteria cause dental diseases such as dental caries, periodontal disease, endodontic disease and pyogenic infection. A variety of microbial flora found in oral cavity is cultivable and non-cultivable. Predominant cultivable microbial flora has been studied in this research. Among all the microorganisms present in the mouth, bacteria are the most predominant as they include both aerobic and anaerobic bacteria (Baveza 2022).

Antibiotics have been used for long to cure infections. But medical science, is facing a major challenge of resistance to antibiotics by pathogens that has a deep impact on health of our society. Resistance against antibiotics have drawn the attention of researchers to develop an approach for patient care. At present, researchers are finding it difficult to prevent resistance in pathogens and are constantly trying to get rid of this problem whose advance antibiotic is being developed. Apart from this, researchers are paying attention to new approaches along with the new antibiotic source, herbal medicine is also being seen as a better option. Herbal medicines are derived from plants and they have been used for centuries. Uses of herbal medicine were common in developing countries. Traditionally, diseases were also treated and prevented by these medicines due to lack of advance medical science facility (Treicher 2021).

Herbal medicines have a such chemical compounds that interfere the function of various pathogens including alkaloids, tannins, glycosides, steroids, volatile oils, fixed oils, resins, phenols and flavonoids that are commonly used in medicine and other application which are obtained from their specific parts of plants such as leaves, buds, fruits, seeds, barks and roots (Gupta et al. 2012). As per report of WHO, 80% people of developing country rely on traditional medicine for their primary health and in 85% medicine, herbal medicines are used (Wang 2020; Treicher 2021).

The trust on herbal medicine is increasing gradually. It means a big part of world's population rely on herbal medicine (Wang 2020). The great diversity of plants represents different sources and type of herbal medicine on broad level. This information grants us a significant role plants as sources of herbal medicine for new herbal medicine in the world. Although, development of resistance diminishes the effect of antibiotics since last few decades. Recently researchers are constantly engaged in the screening

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and discovery of new herbal medicine. That specify the significant role of herbal plants in new herbal medicines (Treicher 2021). Clove (*Syzygium aromaticum*) is the aromatic buds of flower of a tree. It belongs to the family Myrtaceae, widely used as spices and home remedies. It has been reported that clove has antimicrobial activities against numerous types of oral bacteria like *Streptococcus mutans* (Gupta and Prakash 2021).

*Azadirachta indica* is commonly known as "Indian neem" and belongs to family Maleacae. Almost whole tree has medicinal properties. Different parts of neem are used as herbal medicine such leaf, bark fruits seeds and root. The neem is used since ancient time as home remedies. Still, it is known as "village dispensary". In (1992), U.S. National academy of science published a report to confer information about medicinal properties of neem. (Kumar and Parmar 1995; Biswas et al. 2002). There are various ingredients such as ß-sitosterol, quercetin and polyphenolic flavonoids, in fresh leave are found in neem as well as seeds containing azadirachtin and gedunin that have antibacterial and antifungal properties (Sarmiento et al. 2011). It has been observed that recently, researchers are trying to further upgrade the information about the medicinal properties of neem. It also has been observed that neem has antimicrobial properties against cariogenic and pyogenic bacteria (Dhaniya 2011). Additionally, Rajasekaran (2008) revealed medicinal properties of neem as antiviral, antifungal, antibacterial, antiseptic, antiulcer and antipyretic (Gupta and Prakash 2021). Neem and clove have been shown to have a strong antimicrobial effect, which scientists have also confirmed in their research paper. In addition, it has beneficial for human health. Apart from this, it was also observed that people who used routinely are less susceptible for dental disease (Tasanarong and Gupta 2021).

## MATERIAL AND METHODS

Bacterial strains were used in this study including Streptococcus mutans (MTCC 497), *Streptococcus mitis* (MTCC 482), *Streptococcus salivarius* (MTCC 412), *Lactobacillus acidophilus* (MTCC 384), *Staphylococcus aureus* (MTCC96), *Staphylococcus epidermidis* (SMCMB 1084), *Veillonella rogosae* (SMCMB 1121), *Bacteroides fragilis* (SMCMB 1181), *Fusobacterium nucleatum* (SMCMB 1096), *Micrococcus luteus* (MTCC106), *Pseudomonas aeruginosa* (MTCC2581. All strains were revived on nutrient agar medium (NAM) and *Lactobacillus acidophilus* on De Man, Rogosa and Sharpe (MRS) agar medium to obtain fresh culture.

All ingredients were weighed and mixed in distilled water. It was placed it on heated magnetic stirrer for homogenous suspension. Before pH adjustment, the medium was mixed by gently stirring then adjusted the pH by digital pH meter (Systronics, µ system 361) as required. Medium was sterilized in autoclave at 121°C for 15 minutes and 15 ibs. It was cooled to about 50°C. Approximately 15 mL of medium was poured in each sterile polystyrene disposable Petri dish aseptically under laminar air flow chamber, then allowed to cool to solidify. If the culture medium was not to be used at that time, then it was placed in refrigerator at 4°C until used.

Antibacterial susceptibility was done by Disc Diffusion, broth dilution and well diffusion method. Disc diffusion method was followed as per guideline of Clinical Laboratory Standards Institute (CLSI). Muller-Hinton agar medium supplemented with 2% glucose was prepared and adjusted pH at 7.2. Fresh test culture was used for investigation of antimicrobial activities and further, prepared inoculum of 2, 5 x 103 CFU/Ml as compare to McFarland density. 100 µl of inoculum was inoculated on Muller-Hinton Agar medium following spread culture technique. The desired antibiotic disc was placed on culture medium with the help of forceps aseptically. Agar well diffusion method worked on same as that of disc diffusion method. Agar well diffusion method was preferred for investigation of antimicrobial activity of extract of chewing product against isolated oral organisms. 100 µl of inoculum of 2.5 x 103 CFU/mL was inoculated on Muller-Hinton Agar medium following spread culture technique. It was then allowed to absorb inoculum on surface of medium for 4-5 minutes in laminar air flow chamber (LAF).

Wells were cut as per the requirement at equidistance using 6 mm sterile Cork borer. 1250 mg/Ml Stock solution of all chewing extract was used. DMSO was used as solvent for dissolving extract of chewing products. 50 µl of test extract of chewing product was filled in pre-cut wells of 6 mm in diameter on MHA plate which was already inoculated with desired concentration of organism. Inoculated petri plate was incubated at 37°C for 24-48 hrs. Zone of inhibition was measured in mm of diameter by Hi Antibiotic Zone Scale- C PW297 (Hi- Media, Mumbai). Data was recorded as zone of inhibition in mm of average of two independent replicates. It was carried out for investigation of minimum amount able to inhibit growth of test organism. A set of 12 test tube with cap was used. Muller-Hinton broth was used as medium.

In first test tube, 2 mL of double strength medium was taken, which containing 200 mg of herbal extract. In next 2 to 11 test tube, 1 mL of double strength medium was taken in each test tube. Distilled water was taken in 12th test tube as negative control. Now first test tube was properly mixed using micropipette 5 to 6 time for uniform distribution. I mL of the suspension was transferred from the first to the second test tube and again mixed thoroughly and then from the second to the third. This was continued for up to 10 test tubes. I mL of 5 × 103 CFU/mL was inoculated into each test tube. It now showed the expected density of 2.5 × 103 cfu/ml. Test tube 11 was used as a positive control, containing double-strength medium and 1 mL of inoculum was added. Now final volume of each test was 2 mL. All test tube was placed at 37°C in BOD incubator for overnight to 48 hours. Turbidity of each test tube was measured by spectrophotometer.

Based on optical density (OD), inhibitory point was marked and 50 µl of suspension from this marked test tube was spreaded on culture medium with the help of sterile glass spreader. Incubated at 37°C for 24 h and counted the appeared colonies on culture medium. CFU (Colony

Forming Unit) was calculated for each marked and control test tube. This data was used for determination of $MIC_{50}$, $MIC_{80}$ and MBC. $MIC_{50}$ shows a 50% reduction in growth as compared to the positive control and $MIC_{80}$ shows an 80% growth reduction compared to the positive control. MBC (minimum bactericidal concentration) were shown to be completely decreased in growth at the particular concentration compared to the positive control. For the comparative evaluation of the DD and BMD methods, Mean and MIC ranges were calculated for each genus species combination. The diameter of zones of inhibition (in mm) surrounding the antimicrobial disc at 24 and 48 h of incubation was plotted against their respective BMD MICs read after 24 hours and 48 hours of inoculation in the form of Scatterplot. Stoical analyses were made using SPSS software package.

**Table 1. Antimicrobial activity of selected extract of *Azadirachta indica* (neem twig) against isolated significant oral fungal flora by agar well diffusion (AWD) method with broth dilution method (BMD) (given each data is an average of two independent replicates).**

| Test bacterial isolates | Zone of inhibition By well diffusion method (diameter in mm) | Growth inhibition in percent detected by Broth Dilution Method(BDM) at different concentration (mg/mL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 100 | 50 | 25 | 12.5 | 6.25 | 3.125 | 1.562 | 0.781 | 0.390 | 0.195 |
| *S.mutans* | 27 | Ngo | Ngo | Ngo | Ngo | MBC | 83 | 52* | 21 | 15 | Nam |
| *S,mitis* | 26 | Ngo | Ngo | Ngo | Ngo | MBC | 82 | 50* | 20 | 10 | Nam |
| *S.salivarius* | 27 | Ngo | Ngo | Ngo | Ngo | MBC | 82 | 48* | 18 | Nam | Nam |
| *S.aureus* | 26 | Ngo | Ngo | Ngo | Ngo | MBC | 80 | 49* | 18 | Nam | Nam |
| *S.epidermidis* | 22 | Ngo | Ngo | Ngo | MBC | 78 | 49* | 20 | Nam | Nam | Nam |
| *L.acidophilus* | 22 | Ngo | Ngo | Ngo | MBC | 80 | 50* | 20 | Nam | Nam | Nam |
| *V.rogosae* | 21 | Ngo | Ngo | Ngo | MBC | 78 | 49* | 18 | Nam | Nam | Nam |
| *B.fragilis* | 23 | Ngo | Ngo | Ngo | MBC | 82 | 51* | 21 | Nam | Nam | Nam |
| *F.nucleatum* | 25 | Ngo | Ngo | Ngo | Ngo | MBC | 80 | 50* | 16 | Nam | Nam |
| *M.luteus* | 23 | Ngo | Ngo | Ngo | MBC | 80 | 49* | 18 | n.am. | Nam | Nam |
| *P.aerogenosa* | 28 | Ngo | Ngo | Ngo | Ngo | Ngo | MBC | 82 | 50 | 15 | Nam |

Note: - mm= millimetre, n.g.o.=no growth observed, n.am.=no antimicrobial activity, MBC= Minimum Bactericidal Concentration, $MIC_{50}$ =Minimum Inhibitory Concentration that inhibit approximately 50% growth.

## RESULTS AND DISCUSSION

Tooth decay was reported as the most common disease in the oral cavity. *Streptococcus mutans* and *Lactobacillus acidophilus* were observed to be the most common cariogenic bacteria involved in dental caries (Wang 2020). *Azadirachta indica* and *Syzygium aromaticum* were investigated for their antimicrobial activities, Current investigation revealed a strong antibacterial activity (Wang 2020; Marina 2022). Also, it was observed that ethanolic extraction method was much better than aqueous extraction. However, many researchers claim that the constituents of neem like β-bourbonene, β-copaene, β-caryophyllene, β-cadinene, Neophytadiene have antimicrobial activity It also further animal testing should be done (Marina 2022). The present study show that the herbal products used in these studies represent better alternate of antibiotics Herbal products decrease the burden of drug. resistance. These are beneficial as home remedies. Zone of inhibition were observed in mm of diameter around wells containing herbal chewing extract used in this studies. Several researchers also confirmed earlier about their medicinal properties of *Azadirachta indica* (neem) (Marina 2022). Tasanarong (2021) reported that extract of neem has strong antimicrobial against most of oral bacteria (Tasanarong 2021; Marina 2022).

Antimicrobial activities were showed by extract of neem against *Streptococcus mutans* (27 mm) (figure 1.0) *Streptococcus mitis* (26 mm), *Streptococcus salivarius* (27 mm (figure 2.0), *Staphylococcus aureus* (26 mm), (figure 3.0) Streptococcus epidermidis (22 mm), *Lactobacillus acidophilus* (22 mm) (figure 3.0), *Veillonella rogosae* (21 mm), *Bactericides fragilis* (23 mm), *Fusobacterium nucleatum* (25 mm). *Micrococcus luteus* (23 mm), *Pseudomonas aeruginosa* (28 mm) as well as MIC50 clove were recorded1.562 mg/mL for *S. mutans*, 3.125 mg/mL for *S. mitis, S. salivarius, S. aureus, S. epidermidis, B. fragilis* and *P. aeruginosa* along with $MIC_{50}$ for *L. acidophilus, V. rogosae, F. nucleatum* and *M. luteus* were observed 6.25 mg/mL. 6.25 mg/mL. Additionally, MBC was also calculated for all isolates used in this study. MBC for *S. mutans* was recorded 6.25 mg/mL and for *S. mitis, S. salivarius, S. aureus S. epidermidis, B. fragilis* and *P. aeruginosa* 12.5 mg/

mL, respectively. As well as for *L. acidophilus, V. rogosae*, *F. nucleatum* and *M. luteus*, were observed 25 mg/mL (table 1.0 and figure 4.0).

Recently several researchers studied on effect of extract of clove on cultivable oral microbial flora. Gupta (2021) revealed that clove have strong antimicrobial activity against *Streptococcus mutans*. Present study also claims similar study as earlier investigated. Antimicrobial activities were showed by extract of clove like *Streptococcus mutans* (29 mm), (figure 4.0), *Streptococcus mitis* (26 mm), *Streptococcus salivarius* (27 mm), *Staphylococcus aureus* (24 mm), (figure 2.0) *Staphylococcus epidermidis* (23 mm), *Lactobacillus acidophilus* (18 mm) (figure 3.0), *Veillonella rogosae* (18 mm), *Bactericides fragilis* (17 mm), *Fusobacterium nucleatum* (21 mm). *Micrococcus luteus* (22 mm), *Pseudomonas aeruginosa* (24 mm). $MIC_{50}$ were observed as 1.562 mg/mL for *Streptococcus mutans*, 3.125 mg/mL for *Streptococcus mitis, Streptococcus salivarius, Staphylococcus aureus, Staphylococcus epidermidis, Bactericides fragilis, Pseudomonas aeruginosa* respectively. 6.25 mg/mL for *Lactobacillus acidophilus, Veillonella rogosae Fusobacterium nucleatum* and *Micrococcus luteus.* Minimum Bactericidal Concentration (MBC). Gupta and Prakash (2021) revealed that *Syzygium aromaticum* (clove) have antimicrobial activity due to their eugenol derivatives in vitro (figure 4.0) (Gupta 2021).

**Table 2. Antimicrobial activity of selected extract of *Syzygium aromaticum* (clove buds) against isolated dominant oral fungal flora by agar well diffusion method and broth dilution method (given each data is an average of two independent replicates).**

| Oral isolates | Zone of inhibition By well diffusion method (diameter in mm) | Inhibition of growth in percent determined by Broth Dilution Method (BDM) at different concentration .(mg/mL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 100 | 50 | 25 | 12.5 | 6.25 | 3.125 | 1.562 | 0.781 | 0.390 | 0.195 |
| *S.mutans* | 29 | Ngo | Ngo | Ngo | Ngo | MBC | 83 | 52* | 25 | 15 | Nam |
| *S,mitis* | 26 | Ngo | Ngo | Ngo | MBC | 80 | 49* | 18 | Nam | Nam | Nam |
| *S.salivarius* | 27 | Ngo | Ngo | Ngo | MBC | 82 | 52* | 20 | 15 | Nam | Nam |
| *S.aureus* | 24 | Ngo | Ngo | Ngo | MBC | 80 | 52* | 20 | 10 | Nam | Nam |
| *S.epidermidis* | 23 | Ngo | Ngo | Ngo | MBC | 78 | 50* | 18 | Nam | Nam | Nam |
| *L. acidophilus* | 18 | Ngo | Ngo | MBC | 82 | 52* | 20 | Nam | Nam | Nam | Nam |
| *V.rogosae* | 17 | Ngo | Ngo | MBC | 80 | 50* | 20 | Nam | Nam | Nam | Nam |
| *B.fragilis* | 23 | Ngo | Ngo | Ngo | MBC | 80 | 48* | 18 | Nam | Nam | Nam |
| *F.nucleatum* | 21 | Ngo | Ngo | MBC | 83 | 50* | 20 | Nam | Nam | Nam | Nam |
| *M.luteus* | 22 | Ngo | Ngo | MBC | 82 | 52* | 20 | Nam | Nam | Nam | Nam |
| *P.aerogenosa* | 24 | Ngo | Ngo | Ngo | MBC | 80 | 50* | 20 | 10 | Nam | Nam |

Note: - mm= millimetre, Ngo. =no growth observed, n.am.=no antimicrobial activity, MBC= Minimum Bactericidal Concentration, (*) =$MIC_{50}$ (Minimum Inhibitory Concentration that inhibit approximately 50% growth)

**Figure 1: Comparison of Clove, neem, antibacterial activity with Amoxillin, Ofloxacin and Azithromycin antibiotic against *S. mutans***

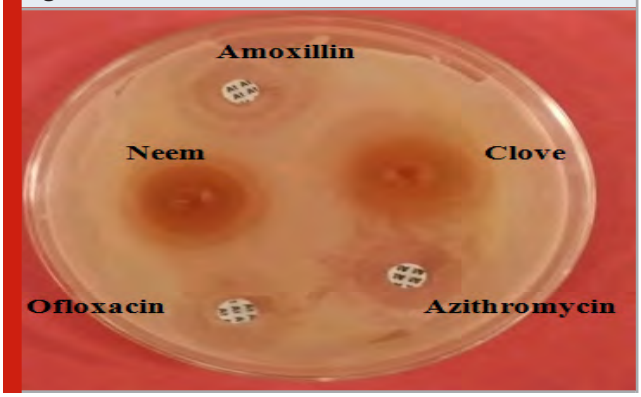



**Figure 2: Antibacterial activity of extract of clove and neem against *S. aureus*, using agar well diffusion method**

**Figure 3: Antimicrobial activity of extract of neem and clove against isolated *L. acidophilus*, using well agar diffusion method**

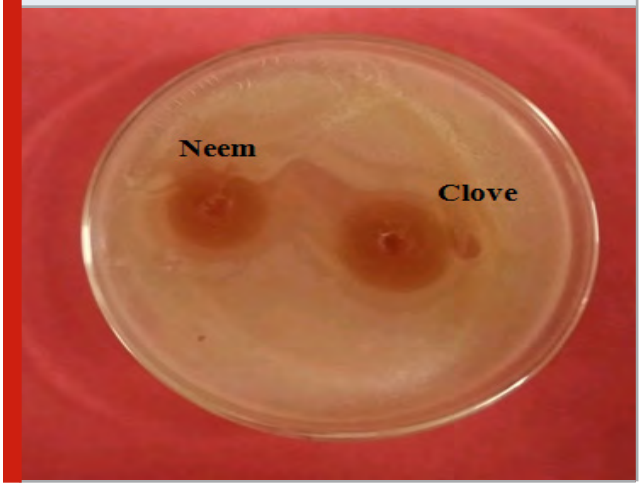
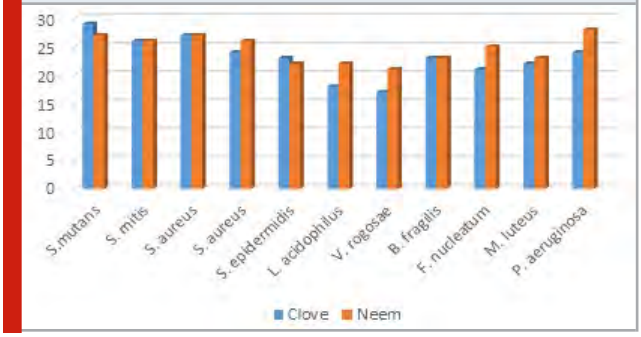



**Figure 4: Antimicrobial activities of clove and neem extract against isolated significant oral bacteria**




## CONCLUSION

The findings of the present studies have confirmed antimicrobial activity of clove and neem against microbial flora. Clove and neem are known for ages for the antimicrobial activities against wide level of microorganisms. These are beneficial when microbes are getting resistant for antibiotic.

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**Medical Communication**

# New Age Vaccines: Technologies in Developing Vaccine Candidates for RNA Virus Diseases

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## ABSTRACT

Vertebrate RNA viruses cause most of the infectious, contagious, transboundary diseases of mammalians in the world. Since 2000, H5N1 avian flu, H1N1/H1N2 Swine flu, SARS, MERS, CCHF, and Covid-19 have caused outbreaks. In addition, rabies, HIV, measles, viral hepatitis, respiratory viruses, dengue, in human beings, and FMD in cattle, PPR in goat, Bluetongue in sheep, infectious bronchitis and PRRS in pigs, are prevalent and endemic in different countries since many years. Whole virus inactivated and live attenuated vaccines including non-pathogenic mutants have been successful in control and eradication of many viral diseases of man and animals. The advancements in molecular virology, non-replicating designer virus vectors of adenovirus and Poxvirus origins, and replicating designer virus vectors derived from vesicular stomatitis virus are being used to deliver immunogenic genes of other viruses to confer protection. RNA vaccine in the form of nucleoside modified mRNA has been successful in control of Covid-19. Single cycle replicon (SCR) virus construct with target transgene, and codon-pair bias deoptimized (CPD) virus have been promising vaccine platforms; both mutants are live attenuated and non-transmissive between host cells. Antigenic spectrum of CPD-virus is as wide as virus attenuated by serial passage in experimental hosts, and is very quick to develop. The technique of synthetic attenuated virus engineering has been faster in developing new age viral vaccines, and also faster to update to match antigenic diversity. This review describes applications of CPD, SAM and SCR technologies in developing vaccine candidates for RNA virus diseases.

**KEY WORDS:** CODON-PAIR DEOPTIMIZATION, NEW AGE VACCINES, SELF-AMPLIFYING MRNA, SINGLE CYCLE REPLICON.

## INTRODUCTION

Vaccines against various viral diseases have been proved to be saviour and guardian of one health domain since decades. Conventionally, two types of vaccines viz., live attenuated and inactivated vaccines have occupied the health sector with their own limitations as they are easy to produce comparatively. Cleverly viral agents have co-evolved in the vaccinated hosts and evaded the host immune mechanism. This has opened the newer areas of concern leading to revisit the vaccinology and produce more sophisticated vaccines using latest molecular biology techniques (Pollard and Bijker 2021).

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With this background, this review is thought of to impart knowledge of new age vaccines to the academicians and researchers. Application of different platforms/ technologies like codon pair bias deoptimized (CPD), self-amplifying mRNA (SAM), single cycle replicon (SCR) vaccines, in developing viral vaccines has been described in detail by (Frederiksen et al. 2020). Still needs to be strengthened in bringing out the products using such technologies towards the use by stakeholders replication-deficient virus are genetically defective in replicating their genome, as in case of current adenovirus vectored SARS-CoV-2 spike gene vaccines, whereas in SCR-virus, the genome replicates using viral RNA replicase- transcriptase to increase copy number of the transgene(s)/ cloned gene(s) so that the target protein molecule is translated in more quantity in the host cells leading to higher immune response in the body, but there is no synthesis of new virion particles due to absence of critical viral gene(s) (Pollard and Bijker 2021).

The SCR virus technology is a quick and precise genomic process in attenuating pathogenic virus for use in vaccines, and en-capsidation (morphogenesis)- defective SCR-Flavivirus has been used as vaccine candidate (Widman et al. 2008; Terasaki et al. 2015). The SCR- virus mutant do not yield new virions, unlike the CPD attenuated virus. Incorporation of underrepresented (rare) codon pairs, but not frequency of CpG di-nucleotide, is the chemistry of attenuation by CPD (Groenke et al. 2020). Of late, RNA vaccines in the form of self-amplifying mRNA (SAM), alternatively self-amplifying RNA (saRNA), built on alphavirus replicon, has found wide application including for control of Covid-19 pandemic Frederiksen et al. 2020; Bloom et al. 2021). Some of the examples of pathogenic RNA viruses modified as vaccine candidates by nucleotide sequence alteration/ deletion technologies, viz., CPD, SAM and SCR are presented in the present review for the benefit of academicians and researchers in virology and vaccinology. Vaccine candidates developed by either of these three new technologies are classified as live attenuated and non-infectious (Pollard and Bijker 2021).

**Genome composition in vertebrate RNA viruses:** The relative occurrence of dinucleotides (two adjacent nucleotides in a polynucleotide chain) is genomic signature of a species Elango et al. 2009). The di-nucleotide TpA is rare (under-represented) in most of the organisms to circumvent nonsense mutations, whereas frequency of occurrence of CpG vary between eukaryotes. Use of CpG in (-) single stranded RNA viruses may not be influenced by the host tropism. In contrast to single stranded RNA viruses and reverse transcribing ones, there is high occurrence of CpG di-nucleotide in dsRNA viruses (Karlin and Mrazek 1997; Cheng et al. 2013).

**CPD attenuated virus as vaccine candidate:** For codon optimization, synonymous replacement of rare codons is made to match with availability of codon specific aminoacyl-tRNA in host cells that enhances protein translation. Inclusion of rare codons attenuated poliovirus (Burns et al. 2006; Mueller et al. 2006), and this attenuation process was named as synthetic attenuated virus engineering (Coleman et al. 2008). Codon pair bias in virus is host species dependent, and virulent virus can be attenuated by synonymous replacement of efficient codon pairs with inefficient/ rare codon pairs in the required viral gene (protein) that is called as Codon-pair bias deoptimization with no alteration in amino acid sequence and composition (Coleman et al. 2008; Broadbent et al. 2016; Kunec and Osterrieder 2016). Codon-pair (bias) deoptimization (CPD) has been effectively used to attenuate virulent viruses (Coleman et al. 2008; Mueller et al. 2010; Broadbent et al. 2016; Groenke et al. 2020).

CPD attenuates viruses as genes re-encoded with rare codon pairs translate less protein due to limitation in availability of required codon specific aminoacyl-tRNAs in host cells; protein formation is controlled by the quantity of mRNA copies and their translatability that is linked to availability of tRNA isotypes (Broadbent et al. 2016; Groenke et al. 2020). The CPD virus is replication competent, antigenically identical to the parent strain, and induce specific immune response akin to the virulent strain (Coleman et al. 2008; Mueller et al. 2010; Broadbent et al. 2016).

**Self-amplifying mRNA/RNA (SAM/saRNA) as vaccine candidate:** During the last about two decades, vaccine development platform using synthetic RNA has been adapted with success; mRNA vaccines for preclusion of infectious diseases have been a substitute for conventional vaccines, with the additional advantage of being cold-chain independent (Maruggi et al. 2019; Bloom et al. 2021). The RNA/mRNA vaccines are commonly formulated on RNA genome of single stranded positive sense RNA viruses (Maruggi et al. 2019; Bloom et al. 2021). RNA vaccine platform using synthetic alphavirus replicon that has 5′ cap, NSP1–4, 26S sub-genomic promoter, and 3′ poly A tail, but no structural protein genes, known as self-amplifying mRNA (SAM)/ self-amplifying RNA (saRNA) technology, has been promising (Zhou et al. 1994; Geall et al. 2012; VanderVeen et al. 2012; Geall et al. 2012; Luis et al. 2015; Samsa et al. 2018; Ballesteros-Briones et al. 2020). RNA vaccines on alphavirus derived SAM stimulates innate immunity through pattern recognition receptors (PRRs), and also elicits strong and specific humoral and cellular immune responses (Yoneyama et al. 2010; Ulmer et al. 2012; Atasheva et al. 2012; Maruggi et al. 2019; Ballesteros-Briones et al. 2020). SAM vaccines using influenza virus HA, human cytomegalovirus glycoprotein gB, HIV envelope glycoprotein, respiratory syncytial virus F antigen, malaria- plasmodium protein PMIF, rabies virus G protein have been developed and evaluated (Geall et al. 2012; Brazzoli et al. 2015; Brito et al. 2015 Bogers et al. 2015; Baeza Garcia et al. 2018; Stokes et al. 2020).

**Single-cycle replicon (SCR) virus as vaccine candidate:** Application of SCR technology in developing vaccine candidate for RNA virus diseases and also for ds-DNA viruses like ASFV and HSV is described (Freitas et al. 2019; Ramsey et al. 2020).

**(i) Single-strand segmented negative sense RNA virus:** Rift Valley Fever virus (RVFV): Rift valley fever (RVF) is a zoonotic disease primarily of domestic ruminants and humans with an incubation period of 2-6 days (WHO) that was first documented in 1931 in the Great Rift Valley of Kenya. The RVFV is an insect-borne enveloped virus (Fig:1) that is maintained between ruminants and mosquitoes. Humans get infected by the RVFV either by mosquito bite or contact with materials contaminated with the virus. The virus to the genus Phlebovirus, under family Phenuiviridae and order Bunyavirales, and has 3 segments of negative sense RNA genome (11.5 kb), viz., L, M, and S (Walter and Barr 2011; Nuss et al. 2014) (Fig: 2). The genus phlebovirus has more than 60 species (ICTV, 2021). As the RNA genome segments are of negative sense, the complete virion particles carry molecules of L and N proteins that are required for the initial replication/ transcription of viral RNA (Nuss et al. 2014).

**Figure 1: Structure of RVF virus. vRNP- viral ribo-nucleoprotein, three in number; N- nucleoprotein; L- viral polymerase; Gn and Gc- virus surface glycoproteins.**

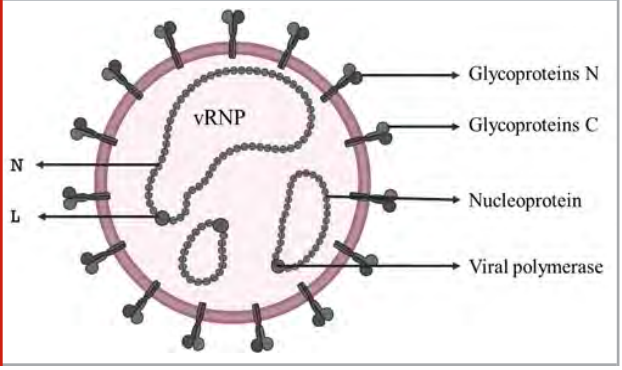
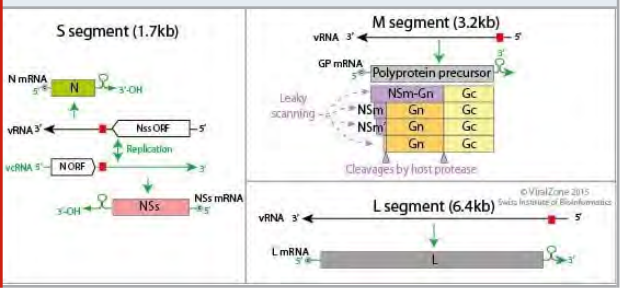



**Figure 2: Genome organisation of RVF virus (Source: Alchetron: Phlebovirus)**



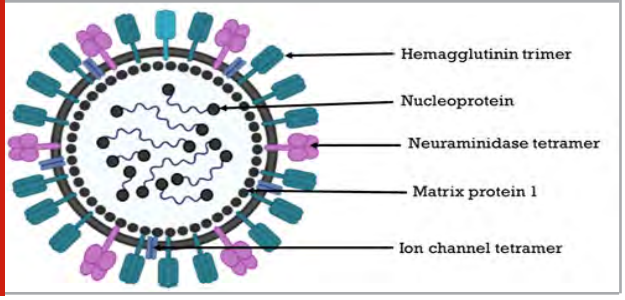

Immunization with RVF-VLP carrying N gene protected mice (Pichlmair et al. 2010). VLPs are devoid of viral genome, whereas SCR virions are genomic mutant. SCR-RVFV elicited protective immunity without side effects in hosts (Terasaki et al. 2015). This SCR-RVFV mutant had L and S RNAs, and devoid of M RNA; absence of M RNA segment (no glycoproteins Gn and Gc) prevented formation of new virus particles. The L and N gene/protein in the SCR-RVFV effected single-cycle replication of the mutant RVFV genomes, followed by translation and accumulation of L and N proteins in the infected cells. An SCR-RVFV having NSm, Gn and Gc gene sequences protected mice and sheep (Kortekaas et al. 2012). Another SCR- RVFV carrying nucleotide sequences of L- RNA, N gene, and Gn protected mice and provided sterile immunity in sheep after single dose; Gn glycoprotein elicited high level of neutralizing antibodies in vaccinates (Oreshkova et al. 2013). SCR-RVFV prepared from a mutant strain MP-12 and having L-RNA, M-RNA with mutations $F^{826}N$ and $N^{827}A$ in Gc glycoprotein to eliminate membrane fusion activity of the viral envelope required during morphogenesis of new virions and prevent multi cycle infection, and N gene (S-RNA) protected suckling mice after intracranial challenge with MP-12 RVFV (Terasaki et al. 2015). A new, promising vaccine for RVF is significantly SCR-MP-12 (Caplen et al. 1985; Murakami et al. 2016).

**Influenza A virus:** Influenza A virus (IAV) is the only species of genus Alpha influenza virus in the family Orthomyxoviridae. A, B, C, and D are the four primary influenza viral types. A big strain of influenza A virus infects wild aquatic birds and can spread to other terrestrial species, including humans. IAVs, like all avian influenza viruses (AIVs), are enveloped, pleomorphic, and have eight distinct negative sense RNA genomic regions extending from 890 to 2341 nucleotides (Webster et al. 1992; Klenk et al. 2008). RNA polymerase complex PB2 (cap-binding), PB1+F2 (polymerase) and PA (endonuclease), (HA) hemagglutinin (segment 4), NP (segment 5), neuramidase (NA) (segment 6), M1 and M2 (segment 7) and NS1+NEP (segment 8) are the viral proteins (Fig: 3). Nucleoprotein (NP) coats each RNA genome segment, forming a ribonucleoprotein (RNP) complex with RdRp and many copies of nucleoprotein (NP) (Lo et al. 2018). The hemagglutinin and neuramidase glycoproteins on the virus surface are antigenically diverse, and divide the IAVs into 18 H and 11 N antigenic subtypes, respectively (Joshi et al. 2021). It includes two new subtypes each of hemagglutinin and neuramidase (H17N10, H18N11) lately identified in bats. The Influenza A serotypes that have been approved in humans are, H1N1, H1N2 (endemic in humans, pigs and birds), H2N2, H3N2, H5N1 (bird flu/ avian flu), H6N1, H7N2, H7N3, H7N7, H7N9, H9N2, and H10N7 (Joshi et al. 2021).

In the upper respiratory tract, the virus adheres to sialic acid (SA) linked to galactose molecule by alpha- 2,6 linkage (SA-alpha2,6 Gal), and to SA linked to galactose by alpha- 2,3 linkages (SA-alpha2,3 Gal) in the lower respiratory tract (Shinya et al.2006). Human adenovirus vector platform has been used for influenza A vaccines (Van Kampen et al. 2005; Weaver et al. 2009; Weaver 2014;

He et al. 2015). Adenovirus serotype 6 was engineered to a SCR- virus, having the E1 gene but deleted E3 gene cassette to prevent formation of new virion particles, to express HA gene of influenza A/PR/8/34 virus (Crosby et al. 2017). The expression cassette consisting of HA gene with CMV promoter- enhancer and SV40 poly(A), and was cloned between Ad6 fibre and E4 genes. In Syrian hamsters and cotton rats, the SCR-Ad6-HA construct generated high level of anti- hemagglutinin antibodies (hemagglutination inhibition), equal to 50% protective dose in humans. Cotton rats vaccinated intranasally developed high level of anti-HA antibodies within 21 days, with low viral load in lungs one day post challenge with live A/PR/8/34 virus at 48 days post vaccination. These observations suggest that SCR-adenovirus based vaccines could be suitable for a number of viral pathogens (Crosby et al. 2017). An SCR-Ad6 virus has been used to develop vaccine for Influenza A virus (Crosby et al. 2017; Anguiano-Zarate et al. 2018).

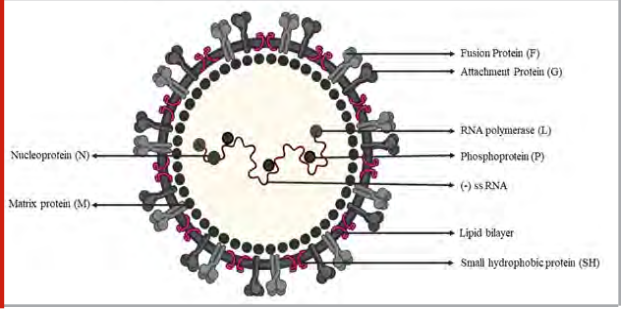
**Figure 3: Structure of Influenza virus (Source: The virology down under blog)**




**2. Single strand negative sense RNA virus:** Respiratory syncytial virus (RSV): Acute infection of lower respiratory tract by RSV is a global human health problem with no licensed vaccine (Mazur et al. 2018). RSV belongs to genus Orthopneumovirus in family Pneumoviridae, order Mononegavirales (ICTV). The virion (120-200nm in size) contains one molecule of negative-sense, single-stranded RNA genome of about 15.2 kb with genome organisation 5'-NS1-NS2-N-P-M-F-M2(1,2)-SH-G-L-3' (Munir et al. 2018; Cifuentes-Muñoz and Dutch 2019). The RNA genome has 10 ORFs coding 11 proteins viz., 5'→3', NS1, NS2, N, M, P, G, F, SH, M2-1, M2-2 and L (Lee et al. 2012; Rincheval et al. 2017). There are two overlapping ORFs in the M2 mRNA that yield two distinct matrix proteins, M2-1 and M2-2 (Borchers et al. 2013). The virus is enveloped with surface glycoproteins F, G and SH, the F and G are essential for virus attachment and fusion (Fig:4). The glycoprotein F mediates cell fusion to form pathognomonic multi-nucleated syncytium, and antigenically and phylogenetically classify RSVs in to types A and B; the former is more virulent than RSV-B (Jha et al. 2016). The other structural proteins are Nucleoprotein (N), Phosphoprotein (P), L (viral RNA polymerase) and matrix (M). NS1 and NS2 suppress type I interferon production and signalling (Munir et al. 2018).

RSV replicates in the cytoplasm of host cells, and there is formation of spherical cytoplasmic inclusion bodies. RSV infection causing respiratory disease in younger children was identified in 1956, and since then only two RSV-antibody preparations, RSV-IVIG and palivizumab, have been licensed for prevention (Mazur et al.2018). Difficulty in having an appropriate vaccine has been due to incomplete understanding of the immunology of RSV, and therefore, different vaccine technologies, viz., nanoparticle-based, live attenuated/ chimeric, virus subunit, vector-based platforms have been applied, and different vaccines for neonate, children and adult were developed (Mazur et al. 2018). Formalin inactivated whole virus vaccine had side effects of respiratory disease (Kim et al. 1969; Mazur et al. 2018). Full-length F- glycoprotein- nanoparticle vaccine (Novavax 2015-18) was partially protective in adults (Mazur et al. 2018). So also, the GSK-RSV-F subunit vaccine had the problem of instability of the pre-F antigen. Further, an F glycoprotein subunit vaccine with a TLR4 agonist as adjuvant, was not efficient in eliminating respiratory sickness (Falloon et al. 2017). DPX-RSV vaccine developed using DepoVax technology and SH protein of RSV was promising (Karkada et al. 2010; Schepens et al. 2015).

**Figure 4: Structure and composition of RSV (Source: biggiesboxers.com)**



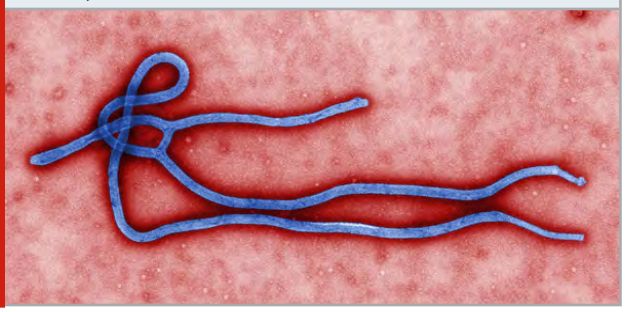

A live attenuated RSV vaccine candidate was developed by CPD (Nouen et al. 2014). Vaccine formulation containing F protein attached to empty bacterial particles was not successful (Van Braeckel-Budimir et al. 2013; Mazur et al. 2018). MVA (modified vaccinia virus Ankara; replication deficient virus) vectored RSV vaccine (MVA-BN-RSV) expressing F, G, N and M2-1 antigens, and replication deficient chimpanzee Adenovirus 155 carrying viral antigens F, N, and M2-1 have been developed and under evaluation. Human Ad26-RSV-PreF vaccine candidate is also under evaluation. VXA-RSV-F, an adenovirus 5 based oral tablet, is expected to circumvent immunosenescence in adult people. Live attenuated RSV with deletion of either M2-2 or NS2 gene elicited strong neutralizing antibodies in children (Karron et al. 2013; Karron et al. 2015). The rBCG-N-RSV chimeric vaccine expressing nucleoprotein of the virus elicited specific antibody and Th1 response essential for protection of lungs (Rey-Jurado et al. 2017). Recently, a (deletion) mutant SCR-RSV strain lacking matrix (M) protein gene was developed (Schmidt et al.

2018). The M protein is essential for new virion assembly/ morphogenesis. Infection with RSV M-negative mutant do not produce M protein, and therefore no new virion is generated during replication. The SCR-RSV lacking M gene, induced robust serum antibody and memory T cell response in mice. This SCR-RSV is a promising live attenuated vaccine candidate, as it provided protection in mice against live virus challenge and reduced virus replication in lung (Schmidt et al. 2019).

**EBOLA virus (EBOV):** The genus Ebolavirus belongs to family Filoviridae in the order Mononegavirales (ICTV). Virion appears filamentous under electron microscope (Fig:5), and contains one molecule of single-stranded negative-sense RNA. There are 7 genes, flanked by UTRs at both the ends. The virus genome is organised as: 3'-UTR-NP-VP35-VP40-GP-VP30-VP24-L-5'-UTR (Feldmann and Geisbert 2011; Schmidt et al. 2019).

Ebola virus disease (EVD) is a highly fatal viral haemorrhagic fever that was identified in 1976 in Sudan and Congo, near the Ebola River (WHO 2014). The virus spreads through infected body fluids including blood, and incubation period varies from 2-21 days. Four of the viruses in the genus Ebolavirus, viz., Bundibugyo virus, Sudan virus, Taï Forest virus, and Ebola virus are associated with Ebola virus disease (Hoenen et al. 2012). Genome back bone of both negative-sense single stranded RNA virus (vesicular stomatitis virus; VSV) and double-stranded DNA virus (ChAd3) were used to clone glycoprotein of EBOV; leading to replication competent rVSV-EBOV and replication defective ChAd3-EBOV vaccine candidates (Holbrook 2018; Samai et al. 2018; Suder et al. 2018).

**Figure 5: Electron micrograph of Ebola virus (Source: CDC/Cynthia Goldsmith - Public Health Image Library, #10816).**



The rVSV-EBOV is a live chimera in which G protein of VSV is replaced with EBOV-G protein. As the ChAd3-EBOV vaccine candidate was replication deficient, there was limited production of the target EBOV-G protein. Both the vaccine candidates were efficacious, but the rVSV-EBOV vaccine candidate caused severe side effects in recipients during clinical trial. MVA-EBOV-G recombinant vaccine candidate could be a booster for ChAd3-EBOV vaccine (Stanley et al. 2014; Holbrook 2018). A SCR-Adenovirus 6 platform carrying EBOV-G protein elicited strong humoral antibody response in mice, hamsters and rhesus macaques, and protected them from rVSV-EBOV challenge (Anguiano-Zarate et al. 2018).

**3. Single- strand positive sense RNA virus: Flavivirus**: The genus Flavivirus in the family Flaviviridae have single-stranded (+) sense RNA genome of about 10-11 kb with 5' cap, but no 3' Poly A tail (ICTV). Flavivirus is about 40 nm in diameter with icosahedral capsid, enveloped with surface projections of 5-10 nm, and replicate in host cell cytoplasm (Chambers et al. 1990). The RNA genome is infectious and monocistronic, that is a single polyprotein is translated from the RNA genome that is proteolytically processed by viral and host proteases to form individual viral proteins (Widman et al. 2008). The members of flavivirus genus are Yellow fever, Dengue, Zika, Japanese Encephalitis, and West Nile Virus encephalitis etc that are transmitted by mosquito/ tick. Several subunit vaccines were also developed and used. Purified NS1 of YFV reduced viraemia in rabbits (Schlesinger et al.1986). Viral protein subunit vaccines were satisfactory for Dengue, JE and WNE (Widman et al. 2008). Several virus-vectored vaccines for Flavivirus diseases were developed and evaluated. Immunization with recombinant Vaccinia and Canarypox virus carrying structural and non-structural proteins of Flavivirus elicited protective immune response in laboratory animals and rhesus monkey (Bray et al. 1989; Yasuda et al. 1990; Konishi et al. 1998; Raengsakulrach et al. 1999; Iglesias et al. 2006).

Lentivirus (positive sense RNA virus) vector carrying trE gene of WNV was protective against live virus challenge after vaccination with 50 recombinant virus particles (Iglesias et al. 2006). Measles virus (negative sense RNA virus) vector carrying E gene of WNV elicited strong neutralizing antibody response and spared mice from live virus challenge (Despres et al. 2005). Replication-defective Adenovirus vectors have been used to express flavivirus antigens, and virus vector carrying NS-1 of TBEV protected > 50% of mice (Jacobs et al. 1994). Adenovirus carrying E gene of Dengue virus-2 elicited neutralizing antibodies in mice (Jaiswal et al. 2003). Adenovirus vector carrying DIII of E gene of both Dengue virus-2 and 4 elicited virus neutralizing antibodies in laboratory animals (Khanam et al. 2007). A formulation carrying two Adenovirus recombinant vectors carrying M and E genes of four serotypes of Dengue virus elicited virus neutralizing antibody response against all the 4 serotypes (Raja et al. 2007; Holman et al. 2007). So, the platform was successful for WNV (Schepp-Berglind et al. 2007). DNA based vaccines for Flavivirus diseases were developed and evaluated (Widman et al. 2008). Plasmid carrying M and E genes of Dengue virus- 2 elicited virus neutralizing antibody response in mice (Konishi et al. 2000; Widman et al. 2008).

DNA construct carrying either M and E genes or NS-1 of JE virus protected 70-90% of mice (Lin et al. 1998). Simultaneous administration of DNA vaccine carrying M and E genes of WNV, and formalin inactivated WNV vaccine had synergistic effect on neutralizing antibody response (Ishikawa et al. 2007). Multivalent recombinant plasmid/ DNA carrying M and E genes of the four Dengue virus serotypes elicited neutralizing antibody response against all (Konishi et al. 2006; Raviprakash et al. 2006). A DNA vaccine for WNV carrying M and E genes was effective in mouse and horse and turned out to be the first DNA vaccine licensed for use in animals (Davis et al. 2001; Widman et al. 2008). Dengue virus attenuated by deletion of a part in the 3'UTR elicited neutralizing antibodies in human and non-human primates (Durbin et al. 2001). Flaviviruses have the potential to exchange structural genes amongst members of the same genus (Widman et al. 2008). The C-prM-E regions of Dengue virus 1 and 3 was cloned in Dengue virus 4 backbone, and the live chimeras were more attenuated than the parent virus (Bray and Lai 1991). ChimeriVax vaccine platform carrying M and E genes of the virus cloned in YFV-17D backbone has been promising (Widman et al. 2008).

The M and E genes of JEV were cloned in YFV-17D backbone (Chambers et al. 1999). This chimera, ChimeriVax-JE, was immunogenic and safe in humans (Monath et al. 2002). ChimeriVax-Dengue was constructed for all Dengue virus serotypes, and ChimeriVax-Dengue-2 elicited strong neutralizing antibody response (Guirakhoo et al. 2006). Viral E protein of WNV was mutated to reduce neurovirulence in mice, and mutated ChimeriVax-WNV (ChimeriVax-WN02) was constructed that protected immunized monkeys from viraemia (Arroyo et al. 2004). SCR- TBE virus was designed by deletion in the Capsid (C) gene of the virus; this attenuated mutant (Kofler et al. 2004) as well as synthetic viral RNA with same deletion elicited defensive immunity in adult mice (Aberle et al. 2005).

This is the first report of RNA vaccine in the form of SCR-Flavivirus. It was shown that YF and WN virus genome with large deletion in the capsid gene can be packaged in host cells expressing the capsid protein from another related virus replicon (Mason et al. 2006). RepliVAX is a Capsid gene deletion mutant virus that replicated and produced progeny virions in cells expressing capsid protein from a replicon of the Venezuelan equine encephalitis (VEE) virus (genus Alphavirus; Togaviridae). However, the mutant genome may replicate once (SCR) in other cells without supplementing the missing capsid protein. RepliVAX-WNV vaccine was protective in mice and hamster (Widman et al. 2008). A RepliVAX- JEV chimera was constructed to prevent JE (Ishikawa et al. 2008).

**Alphavirus:** Members of Genus Alphavirus, family Togaviridae, carry positive sense RNA genome of 11-12 kb with 5'Cap and 3' Poly A tail (ICTV). The virus is enveloped with icosahedral capsid core, and about 70nm in diameter. There are 2 ORFs in the viral RNA genome; the ORF 1 is towards 5' end of the genome that codes for four non-structural proteins, NSPs1-4, whereas the ORF2 is located towards the 3' end of the genome and codes for structural proteins, viz., capsid (C), E3, E2, 6K and E1. The E3 and E2 originate from the precursor protein P62.

**There are three subgroups in the genus Alphavirus;** Semliki Forest virus subgroup, eastern equine encephalitis virus subgroup (EEEV and VEEV) and the Sindbis virus subgroup (Levinson et al. 1990). VEEV is a mosquito-borne viral pathogen that affect all equine species, and also is an emerging zoonotic agent. There are 6 different subtypes, I to VI, of VEEV. The available vaccines include, TC-83 live attenuated and C-84 formalin inactivated vaccines. These vaccines have side effects. Viral RNA genome vaccine platform stimulates innate/ non-specific immune system and antigen expressed by RNA vaccine elicits strong and specific immune response (Ulmer et al. 2012; Atasheva et al.2012). The alphavirus replicon technology (that gave rise to SAM technology) has been utilised in the development of vaccination for animals and man (VanderVeen et al. 2012).

RNA vaccine platform using SAM was developed that used a 9 kb RNA in lipid nano particles (Geall et al. 2012). This SAM construct was a synthetic RNA representing Alphavirus genes coding for RNA replicase/ transcriptase but lacked structural protein genes. This construct included 5′ cap, NSP1–4, 26S sub-genomic promoter, the target gene/protein/antigen (transgene), and 3′ poly A tail. SAM vaccine with VEEV strain TC-83 genome devoid of capsid gene was developed (Samsa et al. 2018). This synthetic SAM at 100µg dose elicited virus neutralizing antibodies, similar to those elicited by TC-83 live attenuated VEE vaccine, and provided thorough protection in mice. This VEE-SAM vaccine is live-attenuated, and undergoes RNA amplification without production of new virion (Samsa et al. 2018).

**Coronavirus:** Spike gene of Infectious Bronchitis Virus (IBV) was found to be a candidate for new IBV vaccine, as Thymidine Kinase deficient recombinant vaccinia virus carrying Spike (S) gene of IBV elicited virus neutralizing antibodies in mice (Tomley et al. 1987). This has led to Spike gene-based vaccines for SARs-CoV-2, TGEV, and CCoV (Torres et al. 1995; Qiao et al. 2005; Yuan et al. 2015; Frederiksen et al. 2020; Lu et al. 2021). Recombinant NDV, LaSota strain, having complete S gene of IBV, cloned between phosphoprotein (P) and matrix (M) genes of NDV, protected chicks against IBV (Abozeid et al. 2019; Jbeli and Jelassi 2021).

Virus-independent entry/ delivery of nucleic acids in to host cells has advantage over virus vectored delivery system (Geall et al. 2012). Replication-deficient virus, whose

genome does not replicate upon infection of susceptible host cells, has been used to formulate different viral vaccines. The gene insert/ transcription unit/transgene is transcribed and translated, and as the recombinant viral DNA does not replicate the antigen/ protein yield could be limited. In contrast, single cycle replicon (SCR) virus, the genome of which replicates with no progeny virion formation, has been used in vaccine design. In both the cases, the mutant virus is classified as live attenuated, and non-infectious as the mutant(s) cannot spread in the body of the host, and whole virus can be rescued only in cells supplementing the missing viral component.

The SCR- mutant based vaccine has the capability and advantage, over replication-defective virus vaccine, of multiplying the viral genome and multiple genome copies produced (by SCR mutant) would lead to higher number of transcribed mRNAs leading to translation of high level of viral proteins, and thereby higher immune response in the host (Widman et al. 2008). Therefore, SCR- vaccines would be superior to replication-deficient vaccines, and can be designed for both RNA and DNA viruses. Further, SCR- vaccine is an easier and efficient platform for positive-sense RNA viruses. The principle of codon-pair bias deoptimization (CPD) by synthetic attenuated virus engineering (SAVE), has revolutionized virus attenuation. Quick development of attenuated virus strains by CPD-SAVE has facilitated faster development of live virus vaccines.

Pathogenic viruses attenuated by CPD can be used as vaccine candidates as such, or can be further modified (recombination) by insertion of an alien gene/ transcription unit (vaccine target) in the inter-genic space available (e.g. between phosphoprotein and matrix genes, and Nucleoprotein and phosphoprotein genes etc) in negative-sense single stranded RNA viruses, viz., morbillivirus, orthoavulavirus and vesiculovirus etc (Brandler et al. 2007; Kasama et al. 2011; Mok et al. 2012; Jbeli and Jelassi 2021). Live-attenuated Schwarz strain of Measles Virus developed by passage in primary human kidney and amnion cells, and then in CEF cells can be used to express heterologous antigens and is being used to develop measles virus- based Covid-19 vaccine (Combredet et al. 2003; Boisgerault et al. 2013; Frederiksen et al. 2020).

Recombinant Measles virus-based SARS-CoV-2 vaccine candidate carrying full length Spike gene of SARS-CoV-2 were found highly efficacious (Horner et al. 2020; Lu et al. 2021). Avirulent Newcastle disease virus (NDV; Orthoavulavirus, Paramyxoviridae) carrying Spike gene of infectious bronchitis virus (IBV; coronavirus) was protective in chicks (Abozeid et al. 2019). The CPD technique is also being applied to develop live attenuated vaccine candidates for SARS-CoV-2 (Frederiksen et al. 2020). The SAM/saRNA vaccine technology using SCR-Alphavirus replicon/ vector has been a success in different virus models including SARS-CoV-2 on VEEV replicon (Zhou et al. 1994; Geall et al. 2012; Vander Veen et al. 2012; Sandbrink and Shattock 2020; Bloom et al. 2021). The transgene (target gene/ transcription unit) is amplified by the Alphavirus RdRp complex for higher antigen/ protein production without formation of progeny virion; the SAM vaccine is also classified as live-attenuated. These are usually delivered encapsulated in lipid nano particles (Geall et al. 2012; Bloom et al. 2021).

## CONCLUSION

The findings of the present review have shown that the development of RNA virus vaccine candidates on RNA virus backbone, e.g., (1) attenuated negative-sense single stranded RNA virus (e.g., measles virus, NDV, VSV etc) carrying a transgene, (2) CPD virus designed by SAVE, and (3) SAM/saRNA molecule designed on alphavirus replicon, is gaining wide application as these vaccine technologies/ platforms are easier to adapt, and such new age viral vaccines can be development in less time, compared to whole virus vaccines, both inactivated and live attenuated, and DNA virus (Adenovirus and Poxvirus) vectored vaccines.

**Conflict of Interests:** Authors declare no conflict of interests to disclose.

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**Biotechnological Communication**

# Effectiveness of Pseudomonas and *Alcaligenes* sp. on the Yield and Lignin Deposition of Barley *Hordeum vulgare* L. Crop.

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## ABSTRACT

The study was conducted to examine the effect of plant growth promoting rhizobacteria; *Pseudomonas punonensis* LMT03 (R1), *Pseudomonas plecoglossicida* (R4), *Pseudomonas aeruginosa* DSM 50071 (R2), *Alcaligenes faecalis* (DBHU5) and their consortium on yield and lignin deposition of the barley crop. Consortium treated plants had the highest plant height, leaf surface area, number of fertile tillers, spike length, grains per spike, 1000 grain weight, grain yield, straw yield, total biomass, and harvest index percent and the lowest values were found in control plots. The consortium treated plant produced the highest grain yield 7976 kg/ha, while control plants produced 3200 kg/ha. In comparison to the control plant, the PGPR-treated barley plant showed dense lignin deposition in the vascular bundles of the stem section.This is the first report on the effect of *P.punonensis* and *P. plecoglossicida* on barley crop yield parameters under field conditions, also the first report on lignin deposition in barley plant treated with *P. punonensis* and *P. plecoglossicida* strains.

**KEY WORDS:** BARLEY, GRAIN YIELD, INOCULATION, LIGNIN, PLANT GROWTH PROMOTING RHIZOBACTERIA.

## INTRODUCTION

Barley (*Hordeum vulgare* L.) is a member of the Poaceae grass family, ranking fourth among cereals behind maize, wheat and rice with respect to its worldwide production. It is the fast growing annual crop, grows in winter season (Ghanbari et al. 2012). Barley is a diploid species with a high degree of inbreeding (Self pollinating) capability. It has a low chromosome number (2n=14) with a large genome size, can be easily cultivated and adapt to different climatic and environmental conditions; and easily cross-bredable. Because of its adaptability and robustness, barley is grown in over 100 countries worldwide (Harwood 2019). Barley crops were used in the brewing industry, as animal feed, and as healthy food options for human consumption. They were also used as a cover crop to increase soil fertility (Ghanbari et al. 2012).

The inclusion of barley in the diet provides several health benefits, including lowers blood cholesterol levels, increased fibre intake, and a good source of beta-glucan, the highest amount of beta-glucan was found in barley (Behall et al. 2004; Harwood 2019).

The amount of grain produced is insufficient to meet the food demand of India's uncontrolled growing population; thus, to increase crop productivity and yield to feed the growing population, synthetic fertilizers and pesticides were incorporated into farming without regard for the negative and hazardous impact on the environment, food chain, and human health. The uncontrolled use of chemical fertilizers in agriculture is presently the topic of debate due to environmental concern and fear for living being health (Turan et al. 2010). The synthetic chemical fertilizers are the inorganic fertilizers rich in major nutrients NPK (nitrogen, phosphorus, potassium) in huge amount and do not incorporate organic manures consequently results in deterioration of soil quality and its fertility (Choudhry 2005; Harwood 2019).

Plant growth promoting rhizobacteria (PGPR) were the healthy and cost effective strategy toenhance the crop productivity. Application of PGPR as biofertilizer are the most effective approach to enhance the sustainable agricultural systems (Sharma 2003). PGPR are soilborne bacteria that aggressively colonize the rhizospheric region of plants or when applied to the seeds or crops enhances the

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growth and yield of plants (Kaymak 2011). Incorporation of PGPR instead of chemical fertilizers are known to improve grain yield through supply of plant nutrients may help to sustain and protect environmental health (O'Connell 1992). Inoculation of phytomicrobiome members in agriculture for crop productivity is a sustainable and cost-effective approach to disease control and artificial,chemical, synthetic supplements could reduce the negative effects associated with the excessive use of chemical fertilisers and pesticides (Antar et al. 2021b). These phytmicrobiomes have been used as an effective strategy to reduce biotic and abiotic stresses that could improve crop productivity (Khan et al. 2020; Antar et al. 2021b).

Lignin is the second richest biopolymer of high molecular weight having complex phenolic structure and called as major structural component of plant cell (Nayak et al. 2020). Lignin deposition in the cell wall is the important step during stem development, provides strength to stem which is interlinked to barley growth, agronomic traits and hence affects yield (Jones et al. 2001; Begović et al. 2015). Lignin deposition also play crucial role in water and mineral transport, activates defence mechanism against biotic and abiotic stresses, provides rigidity and mechanical strength to the tissues through thickening of plant cell wall and development of secondary growth, helps in plant tissue/ organ growth and development, imparts culm lodging resistance and many more in favour of plant metabolism (Jayamohan and Kumudini 2011; Liu et al. 2018).

It was found that lignin deposition in monocot plant such as barley is not very much intensive and thus have not that much intense secondary growth in comparision to dicot plants, in which the secondary cell wall is made up of 20% of lignin (Vogel 2008). Lignin deposition protects the monocot plants such as barley from poor culm strength which is also termed as culm lodging (Hai et al. 2005; Ma 2009). Moreover, lignin deposition generate resistance against culm or stalk bending (reduction in culm height), imparts strength to the barley culm which have direct impact on grain yield as bending of culm or reduction in culm height leads to shorter plant height which consequently reduces the grain yield (Lalić et al. 2005; Bonawitz and Chapple 2013).

It was reported that PGPR treated plants can enhances the lignification in plants (Jha 2019). Increased lignin deposition were found in *Azospirillum* brasilense treated strawberry plant which also provide resistance against charcoal rot disease (Viejobueno et al. 2021) Maximum lignin deposition was found in vascular bundle of chickpea plant inoculated by fluorescent *Pseudomonas* and *Rhizobium* PGPR strains (Singh et al. 2013; Viejobueno et al. 2021).

PGPR application as biofertilizer in plant is a sustainable approach to improving crop production. Employment of PGPR can be introduced to achieve the pupose of achieving the sustainable and resilient agricultural production system without application of additional chemical fertilization. In the view of above background information the present investigation was aimed to evaluate the efficacy of PGPR isolates; *Pseudomonas punonensis* (R1), Pseudomonas *plecoglossicida* (R4), *Pseudomonas aeruginosa* (R2), *Alcaligenes faecalis* (DBHU5) and their consortium on yield and yield attributing agronomic parameters such as plant height, leaf surface area, number of fertile tillers, spike length, grains per spike, 000 grain weight, grain yield, straw yield, total biomass and harvest index % of barley plant, further to investigate the effect of these PGPR strains on lignin deposition at the vascular bundle, cell wall of stem section of barley.

## MATERIAL AND METHODS

In order to investigate the impact of PGPR on yield and yield components of barley (*Hordeum vulgare* L.) in the field condition, the barley variety PL-426 were sown during the Rabi season of 2018-19 at Botanical Garden of Banaras Hindu University, Uttar Pradesh, India. The soil of experimental plot was fertile, alluvial loam and is characterized as type of Indo-Gangatic plains. Rabi season is the winter season in the northern India where crop is shown in the month of November-December and harvested in March-April of the subsequent year.

The experimental design was laid out in split randomise block design plot with date of sowing on 1st December 2018, having 2 conditions that are PGPR treated and control (without any treatment of PGPR and chemical fertilizer) having 6 treatments, with 3 replications of each treatment. Seeds were sown in 6m by 3m total area (1m by 1m each plot) having 6 rows and 3 columns,a total of 18 plots. Row to row distance were 20 cm. Seeds were inoculated by PGPR strains by dipping the seeds for 5 hours into the bacterial broth prior to sowing in the field. Maximum precautions were taken to avoid any contamination and mixing of bacterial inoculations during sowing.The field was plowed twice prior to sowing the seed also weeds, unwanted materials were removed and cleaned manually. Plots were irrigated regularly with raw water without any mixture of chemical fertilizers.The crops were harvested during first week of April. The experiment had 6 treatments which are described below

R1- Seeds inoculated with PGPR Pseudomonas punonensis LMT03(Accession no. MT677939)

R4- Seeds inoculated with PGPR Pseudomonas plecoglossicida (Accession no. MT883433)

R2- Seeds inoculated with PGPR Pseudomonas aeruginosa DSM 5007(Accession no. MT845116)

DBHU5- Seeds inoculated with PGPR Alcaligenes faecalis (Accession no. MT872514)

Consortium- Combined treatment of all the 4 PGPRs (R1, R4, R2 and DBHU5)

Control- Without any PGPR treatment and any fertilizer (Non inoculated). Irrigation with raw water only.

For evaluation of yield and yield attributing agronomic parameters ten randomly crop plants were selected from

each of three replicates, all the parameters were recorded from selected plants.Yield was estimated through harvesting all the crop plants of each plot (three replication of each treatment). Mean value of all the three replications of each treatments were considerd for calculation of all agronomic traits. The agronomic parameters studied was: Leaf surface area (cm2) – Leaf surface area were taken by measuring the length and width of a leaf using scale.Plant height (cm)- At physiological maturity, height was measured from the ground level to the top of the spike (excluding the awns) using a meter rod. Number of productive/ fertile tillers- Number of fertile tillers per selected plants were counted.

Spike length (cm)- Three spikes from each of the ten plants per plots selected and length of spikes were recorded from the base to the apex of the spike through a meter rod. Number of grains per spike- Three spikes of each selected ten plants from each replication were threshed and the grains were separated from the spikes and were counted manually. 1000 grain weight (gm) – Thousand grains were counted after harvest and weighed for each replication. After harvest, weight of thousand grains from each plot were taken using weighing balance. The mean value of three replication was used in figure. Grain yield/ (kg/ ha)- After harvesting, grains were threshed and seperated, grain weight of all crop plant of each plot were taken in kg using electronic balance and subsequently converted into kg/ha.

Straw yield (kg/ha)-After harvesting weight of sun-dried above ground parts (excluding grains) of all crop plants from each plots were taken using electronic balance in kg and subsequently converted into kg/ha. Total biomass/ Biological yield (kg/ha) – All crops from each plot under that area were harvested, bundled, sun dried and then weight the bundles in kg using electronic balance for estimation of total biomass , afterward converted into kg/ha. Harvest index%- Ratio between grain yield and total biomass of all crops of each plot was determined by applying the following formula: HI (%) = (Grain yield each replication/ Total biomass (grain + straw) each replication ×100.

**Histochemical deposition and distribution of lignin deposition in barley internodes:** To estimate lignin deposition, a hand cut transverse section of fresh barley internode was mounted on a slide and stained with a solution of 0.5 percent saturated phloroglucinol (w/v) with addition of HCl and observed under an Olympus binocular microscope. The appearance of red-violet colour on the section defined the conformation of accurate lignin staining (Jensen 1962).

**Statistical analysis:** The IBM SPSS Statistics Ver.20 software was used for all statistical analysis and calculations. The statistical data were expressed as the mean of three independent replications, standard error of mean (SEM) of three replicates of each experiment, and thrice repetition data of each replicate, and were interpreted using one-way ANOVA followed by Duncan's multiple range test at the P=0.001 significance level. The experiments in this study were carried out in triplicate, with each experiment being repeated three times using a completely randomised design.

## RESULTS AND DISCUSSION

**Yield and yield attributed agronomic parameters:** Grain yield performance is heavily influenced by agronomic traits. Seeds inoculated with PGPR strains *P. punonensis, P. plecoglossicida, P.aeruginosa, A. faecalis* alone and in combination (consortium) significantly increases plant height, number of fertile tillers, leaf surface area, spike length, number of grains per spike, 1000 grain weight, grain yield, straw yield, total biomass, and harvest index. Performance of the plants on all the studied parameters was superior in PGPR inoculated treatments in comparison to the non-PGPR inoculated plants. The maximum increase in all the agronomic and plant growth promoting parameters was recorded by consortium treated plants followed by *P. punonensis, P. plecoglossicida, P. aeruginosa, A. faecalis*, where as the least value of data was obtained from control plots. For all the field parameters, mean value of all the three replications of each treatments were consider for calculation and yield analysis (Fig. 1).

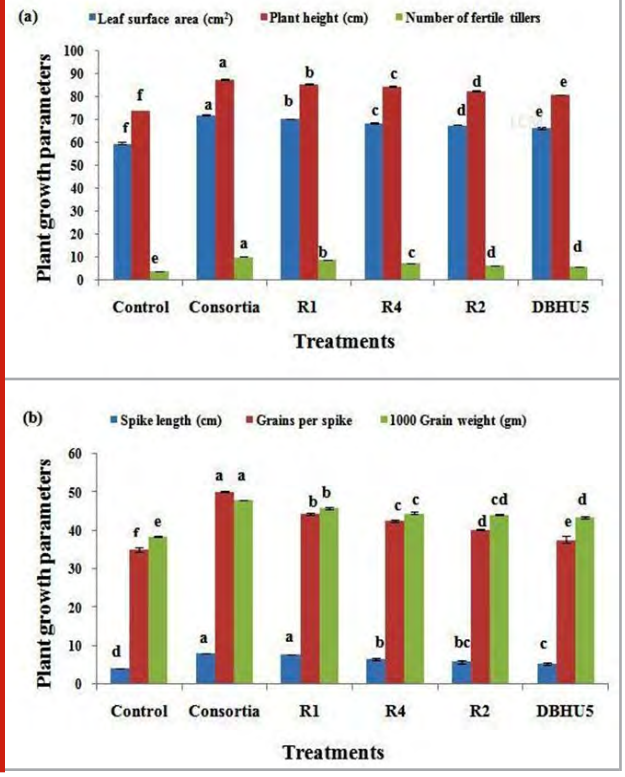
**Figure 1: Barley crop grown in experimental field plot of botanical garden of Banaras Hindu University.**

**Leaf surface area (cm²):** Seeds inoculated with PGPR significantly increases the leaf surface area in barley cultivars over untreated plants. The mean result of all the treatments revealed that the combined effect of all the PGPRs (consortium) produced the highest leaf surface area (72.13 cm², followed by R1 (70.26 cm2), R4 (68.60 cm2), R2 (67.73 cm²) and DBHU5 (66.30 cm2), while the least value (59.73 cm²) was recorded by control treatment. The mean data with respect to leaf surface area have been summarized in Table No. 1 and Fig. 2a. PGPR treatments in barley plants results in increase in leaf surface area which may enhances the gaseous exchange hence rate of photosynthesis increases which promotes various plant metabolic activities. Purwanto et al. have reported that PGPR inoculation in rice plant can increase leaf surface area upto 91.10 percent compared to control plants (Purwanto et al. 2019).

**Plant height (cm):** All the PGPR treatments had significantly increased the plant height over untreated plants. The mean of plant height was observed to be in the ranges of 76.56-87.53 cm. The highest plant height (87.53 cm) was recorded by consortium followed by R1 (85.36 cm), R4 (84.60 cm), R2 (82.56 cm), and DBHU5 (80.80

cm), while the shortest plant height was showed by control plants (76.56 cm). Result of mean plant height indicated in (Table No. 1, Fig. 2a). Plant height is an important factor and positively correlated with grain yield.Increase in plant height by the inoculation of PGPR indicates that PGPR inoculation in barley plants can increase vegetative growth. Increase in plant height of barley, wheat, corn, by PGPR treatment was already reported (Shaharoona et al.2007; Gholami et al. 2009; Shirinzadeh et al. 2013; Hussain et al. 2020).

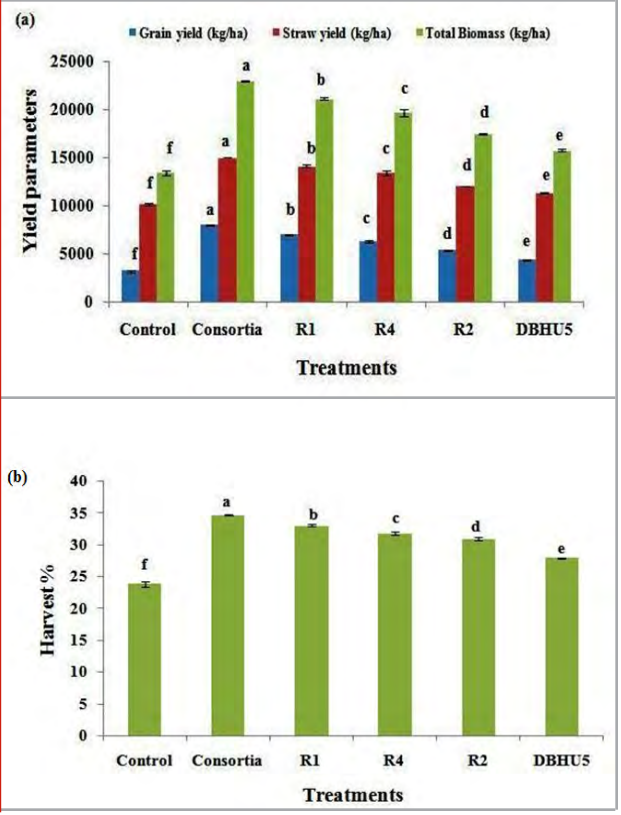
**Figure 2: (a-b) Graph representing effect of different PGPR strains and their consortium on (a.) leaf surface area, plant height, number of fertile/productive tillers, (b.) spike length, grain per spike, 1000 grain weight of barley plants in comparision to control plants, illustrating increase over control in field condition. Data are means of three replicates along with standard error of mean bars. Different letters above the standard error bars denotes significant differences over control (p<0.001).**




**Number of productive/fertile tillers:** Seeds treated by consortium produced maximum number of productive tillers (10.23), followed by R1 (8.76), R4 (7.40), R2 (6.30) an DBHU5 (6.00), the reduced number of productive tillers was produced by control plants.The average number of total fertile tillers as indicated in (Table No. 1, Fig. 2a).The number of fertile tillers is an important agronomic factor that influences grain yield. Similar reports were foundby Shaharoona et al. in wheat crop as the number of fertile or productive tillers in plant increases the number of spikes along with grains, which play a vital role in grain yield (Shaharoona et al. 2007). Increase in number of tillers is one of the chief agronomic character as this may compensate the difference in number of plants, partially or totally after crop establishment and may allow crop recovery from early frost (Acevedo et al. 1998; Hussain et al. 2020).

**Spike length (cm):** The impact of seed inoculation with PGPR treatment on spike length was significant. The maximum spike length (8.10) was obtained by consortium treated plant followed by R1 (7.80 cm), R4 (6.53 cm), R2 (5.86 cm), DBHU5 (5.30 cm) and minimum spike length (4.10 cm) was recorded by control plots indicated in (Table No. 1, Fig. 2b). PGPR inoculated plants showed significant increase in the grain number per spike in comparision to control plants so most highest number of grains per spike shown by consortium (50.10) treated plants, while R1 (44.40) showed the second most highest grains/spike followed by R4 (42.50), R2 (40.23) and DBHU5 (37.63).

**Figure 3(a-b). Graph representing effect of different PGPR strains and their consortium on (a.) grain yield, straw yield and total biomass/ biological yield, (b.) Harvest index % of barley plants in comparision to control plants, illustrating increase over control in field condition. Data are means of three replicates along with standard error of mean bars. Different letters above the standard error bars denotes significant differences over control (p<0.001).**




The lowest number of grain/spike was recorded by control plots (34.96) as indicated in (Table No. 1, Fig. 2b). Increase in spike length, number of grains per spikeis directly proportional to the grain yield, increase in spike length by PGPR treatments, results in more production of grains in spike which consequently results in increase of grain yield of barley plant (Shahzad et al. 2007). In our

study increase in spike length and number of grains per spike through the inoculation of PGPR is according to the findings of Shirinzadeh et al., on agronomic traits of barley (Shirinzadeh et al. 2013). Inoculated barley plants had more grain number per spike and hence more grain yield (Hussain et al. 2020).

**1000 grain weight (gm)-** Based on the data of all treatments of each of the three replicates, the highest 1000 grain weight was recorded by plants inoculated by consortium (47.86 gm), further R1 showed the second highest (45.80 gm), later on R4, R2 and DBHU5 showed (44.50 gm), (44.10 gm) and (43.46 gm) 1000 grain weight respectively. The lowest value was recorded by the grains produced by control plants (38.53) indicated in Table No. 1 and Fig. 2b. 1000-grain weight is an essential yield determining factor of barley. Inoculation of barley seeds with PGPR significantly increases the 1000-grain weight which results in improved seed quality, has been reported by cakmakci et al (cakmakci et al 2007). Increase in 1000-grain weight of barley plant, inoculated by *Azotobacter,Azospirillium, Azotobacter+ Azospirillium* is reported by Shirinzadeh et al. (Shirinzadeh et al. 2013). Wheat plant treated with consortia of *Paenibacillus polymyxa, Bacillus subtilis* and *Bacillus aryabhattai* as well as separate inoculation of these PGPRs significantly increased the 1000 grain weight of wheat (Hussain et al. 2020).

**Table 1. Effect of inoculation with PGPR strains and their consortium on yield and yield attributing parameters of barley crop in comparision to control plants.**

| Treatments | PH | LSA | FT | SL | GPS | TGW | GY | SY | TB | HI |
|---|---|---|---|---|---|---|---|---|---|---|
| Consortia | 87.53± 0.20$^{a}$ | 72.13± 0.08$^{a}$ | 10.23± 0.14$^{a}$ | 8.10± 0.05$^{a}$ | 50.1± 0.05$^{a}$ | 47.86± 0.03$^{a}$ | 7976± 14.52$^{a}$ | 1499± 5.77$^{a}$ | 22966± 20.27$^{a}$ | 34.73± 0.03$^{a}$ |
| R1 | 85.36± 0.27$^{b}$ | 70.26± 0.12$^{b}$ | 8.76± 0.13$^{b}$ | 7.8± 0.05$^{a}$ | 44.40± 0.30$^{b}$ | 45.80± 0.43$^{b}$ | 6976± 14.52$^{b}$ | 1413± 133.33$^{b}$ | 21110± 145.25$^{b}$ | 33.04± 0.17$^{b}$ |
| R4 | 84.60± 0.20$^{c}$ | 68.60± 0.15$^{c}$ | 7.40± 0.05$^{c}$ | 6.53± 0.2$^{b}$ | 42.50± 0.28$^{c}$ | 44.50± 0.28$^{c}$ | 6263± 131.69$^{c}$ | 1343± 260$^{c}$ | 19696± 375.95$^{c}$ | 31.79± 0.26$^{c}$ |
| R2 | 82.56± 0.17$^{d}$ | 67.73± 0.12$^{d}$ | 6.30± 0.24$^{d}$ | 5.86± 0.44$^{bc}$ | 40.23± 0.14$^{cd}$ | 44.10± 0.05$^{cd}$ | 5400± 57.73$^{d}$ | 1203± 33.33$^{d}$ | 17433± 66.66$^{d}$ | 30.95± 0.23$^{d}$ |
| DBHU5 | 80.80± 0.05$^{e}$ | 66.30± 0.30$^{e}$ | 6.0± 0.06$^{d}$ | 5.30± 0.25$^{c}$ | 37.63± 0.91$^{e}$ | 43.46± 0.26$^{d}$ | 4400± 57.73$^{e}$ | 1133± 88.19$^{e}$ | 15733± 145.29$^{e}$ | 27.96± 0.11$^{e}$ |
| Control | 76.56± 0.28$^{f}$ | 59.79± 0.37$^{f}$ | 4.10± 0.16$^{e}$ | 4.10± 0.05$^{d}$ | 34.96± 0.54$^{f}$ | 38.53± 0.2$^{e}$ | 3200± 115.4$^{f}$ | 10200± 115.47$^{f}$ | 13400± 230.94$^{f}$ | 23.85± 0.44$^{f}$ |

Here 'ha', hectare; 'gm', grams, PH- plant heigh, LSA- leaf surface area, FT- number of fertile tillers, SL- spike length, GPS-grain per spike, TGW-thousand grain weight, GY-grain yield, SY- straw yield, TB- total biomass, HI- harvest index. Different letters on mean±standard error denotes significant differences over control ($p<0.001$).

**Table 2. Correlation (Pearson coefficients) among agronomic parameters of all the six treatments.**

| Parameters | PH | LSA | FT | SL | GPS | TGW | GY | SY | TB | HI |
|---|---|---|---|---|---|---|---|---|---|---|
| PH | 1 | | | | | | | | | |
| LSA | .983** | 1 | | | | | | | | |
| FT | .969** | .943** | 1 | | | | | | | |
| SL | .970** | .947** | .985** | 1 | | | | | | |
| GPS | .953** | .908* | .983** | .957** | 1 | | | | | |
| TGW | .975** | .995** | .951** | .940** | .920** | 1 | | | | |
| GY | .988** | .953** | .985** | .987** | .981** | .947** | 1 | | | |
| SY | .978** | .933** | .984** | .987** | .974** | .926** | .995** | 1 | | |
| TB | .984** | .944** | .986** | .988** | .978** | .938** | .999** | .999** | 1 | |
| HI | .992** | .984** | .948** | .962** | .935** | .972** | .979** | .979** | .960** | 1 |

* Correlation is significant at the $p \leq 0.05$ level

**Grain yield/ Economic yield (kg/ha)-** Grain yield varied between 3200 kg/ha in without treated till 7976 kg/ha in seed treated with PGPR. Maximum grain production was recorded by consortium treated plants (7976 kg/ha) followed by R1 (6976 kg/ha), R4 (6263 kg/ha), R2 (5400 kg/ha), and DBHU5 (4400 kg/ha), while control platts produced only 3200 kg/ha indicated in (Table No.1, Fig. 3a). Grain yield is the main goal of agricultural practices by farmers. Grain yield is one of the significant factor towards yield and yield attributing components. Increased grain yield is directly dependent on increase in number of productive tillers and grain per spike which is also supported by the study of Naeem et al. (Naeem et al. 2018). Enhancement in barley plant growth and grain yield through PGPR treatment is reported in a previous study (Cakmakci et al. 2007). Increase in yield of many cereals crops through application of diazotrophs has been in a previous study (Dobbelaere et al. 2003). Bacteria inoculated plants such as corn, sugarcane, rice increases the yield upto 10 to 30 percent as reported in a previous study (Kloepper et al. 1992). Hussain et al. (2020) reported that application of novel *Bacillus* and *Paenibacillus* species bio-inoculants separately and in combination has a positive influence on yield of wheat crop. Application of *Pseudmonas* spp. and *Burkholderia caryophylli* in wheat plant leads to increase in yield of wheat is reported in a previous study (Shaharoona et al. 2007).

Significant increase in barley grain yield by the application of Azotobacter and *Azospirilliu* is reported by Shirinzadeh et al. (Shirinzadeh et al. 2013). Seed inoculation with *Azospirillum* brasilense significantly affects the yield of barley and wheat as reported in a previous study (Ozturk et al. 2003). Similar result was found by combined application of indigenous PGPR; *B. megaterium, A. chlorophenolicus* and *Enterobacter* on wheat grain yield as reported in a previous study (Kumar et al. 2014). Several studies were found in support of significant increase in grain yield by PGPR inoculated plants (Tiwari et al. 1989). Imran et al. (2015) have reported increase in grain yield in *Ochrobactrum ciceri* and *Mesorhizobium ciceri* inoculated chickpea (PUSA-372) plant (Imran et al. 2015). The maximum increase in grain yield of wheat was observed due to the consortium application of PGPR; *Paenibacillus polymyxa, Bacillus subtilis* and *Bacillus aryabhattai* as investigated in a previous study (Hussain et al. 2020). *Bacillus* spp. significantly increased the grain yields of crops such as fingermillet, maize, amaranth, buckwheat and French bean (Pal 1988; Hussain et al. 2020).

**Straw yield (kg/ha), Biological yield/Total biomass (kg/ha)–** Among all treatments maximum significant straw yield (14990 kg/ha) was recorded in consortium treated plants, followed by R1 (14133 kg/ha), R4 (13433 kg/ha), R2 (12033 kg/ha) and DBHU5 (11333 kg/ha), while lowest straw yield (10200 kg/ha) was obtained in control plants indicated in Table 1, Figure 3a. The maximum biological yield were recorded in consortium treated plants (22966 kg/ha) followed by R1 (21110 kg/ha), R4 (19696 kg/ha), R2 (17433 kg/ha) and DBHU5 (15733 kg/ha) while the lowest biological yield (13400 kg/ha) was obtained from control plots (Table No. 1, Fig. 3a). Biological yield is also an important parameter because farmers were interested in straw in addition to grain (Tigabu and Asfaw 2016).

In our study maximum biological yield or total yield was produced from consortia treated barley plants and similar result was found in a previous study by the treatment of triple combination of PGPR *B. megaterium A. chlorophenolicus* and *Enterobacter* on wheat plant which significantly enhanced the straw yield in field conditions in comparision to uninoculated plant (Kumar et al. 2014). Increase in straw yield, total biomass and harvest index by application of phosphate solublizing bacteria on wheat in comparision to control plants (Turan et al. 2010). Combined effect of *Azospirillum lipoferum, Arthrobacte mysorens* and *Agrobacterium radiobacter* increases the grain and straw yield in 3 barley cultivars (Belimov et al. 1995). Seed inoculation with *Bacillus polymyxa* significantly enhanced total yield in rice and chickpea crops (Tiwari et al. 1989). Harvest index (%)- The maximum harvest index value was obtained from consortium treated plants (34.73%), followed by R1 (33.04 %), R4 (31.79 %), R2 (30.95 %) and DBHU5 (27.96%), while the lowest harvest index was recorded by control plants (23.85%), as explained in Table No. 1, Fig. 3b.

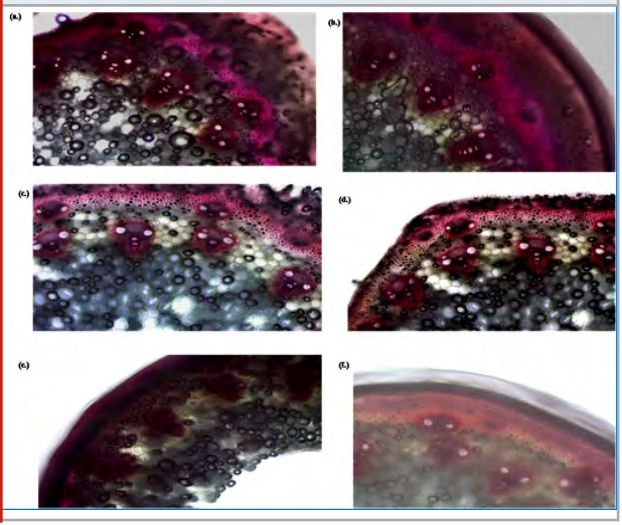
Barley seeds inoculation with PGPR *Azotobacter* and *Azospirillium* enhanced the plant growth promoting parameters such as plant height, spike length, number of spike per area, grains per spike, 1000 grain weight significantly and could enhance grain yield to an acceptable level. PGPR inoculation in crop plants can increase the crop productivity and yield as high as upto approx 57% depending on the crop plant (AsgharMet al. 2004; Shirinzadeh et al. 2013). The PGPR strains; Bacillus megaterium, *Bacillus subtilis,* and *Azospirillum brasilense* were reported to enhance the grain yield, straw yield, total yield, and plant nutrient element content of barley and wheat crop (Baris et al. 2014; Hussain et al. 2020).

**Linear Correlations Between All Agronomic Parameters of all Treatments:** Table No. 2 represents the types of correlations between the agronomic growth and yield parameters of barley. All the 10 agronomic parameters were positively and significantly correlated with each other of all the six treatments, this implies that increase in the value of one parameter leads to increase in the other parameter to which it is significantly correlated and their growth were dependent on each other. As grain yield is our main objective of this study and for the farmers it is the main factor so we recorded that plant height showed highly significant and maximum correlation with grain yield r =0.988**,1Leaf surface area showed r = 0.953** with grain yield, Number of fertile tiller have r =0.985** with grain yield, while spike length r =0.987**, grain per spike r =0.981**, thousand grain weight r =0.947** also showed significant correlation coefficient with grain yield. Here 'r' is correlation coefficient.

From the present correlation data it was concluded that in this study plant height and spike length is positively interlinked with grain yield, if plant height and spike length increases there will be increase in the grain yield also. It was

also observed from the data that if plant height increases there will be increase in the harvest index %. Correlation between different agronomic traits provides necessay information and guidance to the farmers for the selection of yield enhancing traits. In the present study correlation between all the 10 traits; plant height, leaf surface area, number of fertile tillers, spike length, grains per spike, 1000 grain weight, grain yield, straw yield total biomass, harvest index% were analyzed for all the six treatments. All the traits have positive and highly significant interrelationship with each other (Hussain et al. 2020).

**Figure 4. (a-f) Influence of PGPR strains (a.) Consortium (b.) R1, (c.) R4, (d) R2, (e.) DBHU5 on lignification in barley stem by histochemical staining in comparision to stem of (f.) non-inoculated control plant, illustrating increase lignin deposition over control.**



**Deposition and Distribution of Lignin In Internodes of Barley:** Deposition of lignin in pink to violet color were observed in the region of sclerenchyma ring of cortex, epidermis, parenchyma and vascular tissue of all the 4 rhizobacterial isolates (R1, R4, R2 and DBHU5) and their consortium treated barley plants, while there is light or moderate pink color were developed which showed thin or less lignin deposition on sclerenchyma ring of cortex, epidermis, parenchyma and vascular tissue of control plants. PGPR treated plants showed thickness in the cell wall due to lignin deposition in comparision to the non treated plants as observed in Fig 4 (a-f).

As barley is a monocot grass plant which was known for less lignin deposition but in the present result we found that stem vascular bundle section of all the four PGPR treated plant showed intense lignin deposition in comparision to control. Although it was studied and found that lignin synthesis in barley plants occurs with very low intensity and lower quantity, but in the present study it was found that all the four PGPR treated barley plants showed significant and uniform lignin deposition in their vascular bundle region and cell wall of internodes section of stem in comparision to control plants. PGPR treated plants develop maximum cell wall lignification which induces and activates higher concentrations of defense related enzymes, it was found that rhizospheric bacteria Bacillus megaterium enhances the lignin deposition in the cell wall of maize plants and protects against Aspergillus niger (Jha 2019).

Similar result was observed by the treatment of PGPR inoculants *Pseudomonas* and *Rhizobium* on lignin deposition in the vascular bundle of chickpea plant (Singh et al. 2013). Study of the pattern of lignin deposition in the cell wall of internodes of barley (Begovic et al. 2015). Maximum and dense lignin deposition was found in the secondary walls of xylem vessels of strawberry plant treated by *Azospirillum brasilense* (Viejobueno et al. 2021). Lignin deposition in plant stem perform vital role in conductance and movement of water which develop resistance ability in plants under abiotic stress and also provide rigidity to the cell wall (Ajao et al. 2018). Stem and root section of B. megaterium and P. fluorescens treated mungbean plants showed significant increase in lignin deposition which also protect the mungbean plant from the infection of M. phaseolina (Javed et al. 2021).

## CONCLUSION

The findings of the present finding showed significant increase in the yield and yield associated agronomic parameters of barley plants treated by separate inoculation of PGPRs *P. punonensi, P. plecoglossicida, P. aeruginosa* and *A. faecalis* along with combined inoculations of these PGPR strains in comparision to the control plants. PGPR treated barley stem vascular bundle have more intense lignin deposition layer as compared to the non treated plants. All the four PGPR treated isolates showed maximum lignin deposition in the cell wall of barley internodes and consequentialy enhances cell wall thickness in comparision to control. This is the first study on the effect of PGPR; P. punonensis, and *P. plecoglossicida* strains treated barley plants on their yield and yield attributing parameters under field condition. Also this the first study done on characterization of lignin deposition on barley plants treated by P.punonensis and P. plecoglossicida PGPR strains.

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**Declarations**

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in the manuscript and can be shared on a reasonable request made to the corresponding author when required.

**Code Availability:** Not applicable

**Authors' Contributions: KD:** Conceived the research, wrote the manuscript, analyzed the data, acquire the funding. KD: Performed the research. KD: Analyze the data. RSU: Wrote the manuscript, Supervised the research.

**Ethics approval:** Not applicable.

**Consent to participate:** All authors participated in the experiment.

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