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Optimization of Exo-Polygalacturonase Production Activity by Various Factors by Soil Isolate *Bacillus megaterium*

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ABSTRACT

Polygalacturonase represent an important member of pectinase group of enzymes with immense industrial applications that hydrolyzes external and internal α (1–4) glycosidic bonds of pectin to decrease the viscosity of fruits juices and vegetable smashes. Several bacterial strains were isolated from soils of decomposed pectin waste from mango processing industries screened for polygalacturonase production. The strain which produced maximum polygalacturonase was identified as *Bacillus sp*., based on the morphological, biochemical and physiological tests performed which was further confirmed at species level as *Bacillus megaterium* (mpb2) by the molecular characterization with the assistance of IMTECH, Chandigarh, India. Under partially different optimized conditions like temperature, pH, substrate concentration, metal ions, carbon sources, nitrogen sources, surfactants, effect of time and stability of exo-polygalacturonase enzyme activities were studied. Results indicated that the maximum polygalacturonase production by *B. megaterium* were at pH (0.96 U/ml) range of 6.0 to 8.0 and temperature (0.97 U/ml) of 55°C and substrate concentration of 1% citrus pectin (0.73 U/ml). Lactose (1.5 U/ml) as carbon source, Peptone (0.66 U/ml) as nitrogen source, Triton X-100 (0.37 U/ml) as surfactant, $CaCl_2$ (0.25 U/ml) as metal ion, effect of time for 15 minutes at 55°C (0.73 U/ml) and the enzyme stability for one hour at 65oC was (0.73 U/ml) were observed as optimum for the polygalacturonase production by *B. megaterium* respectively. The main objective is to study the various optimum factors on production of exo-polygalacturonase by *Bacillus megaterium* isolated from soil samples collected from pectin industry waste of Vinsari and Varsha mango fruit processing industries around Tirupati, Chittoor district of Andhra Pradesh.

KEY WORDS: EXO-POLYGALACTURONASE, BACILLUS MEGATERIUM, METAL ION, OPTIMIZATION, SURFACTANTS,.

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INTRODUCTION

The microbial enzyme production had greatly influenced by environmental and nutritional factors. Therefore, the selection of microbial source (wild type, recombinant, mutagenized) along with variations in various parameters; pH, metal ions, temperature affects the polygalacturonase production. Surfactants such as tween-20, Tween - 80 increase the enzyme production due to favourable effect on cell membrane permeability which leads to secretion of the enzyme. Pectinase synthesis is inhibited by SDS PAGE because of the denaturation of enzyme. The degrading ability of the enzyme is enhanced by agitation (Ahlawat et al., 2009). The purified polygalacturonase characterization is an important that used for research since it exhibited on being able to distinguish between the enzymatic complex components of the substrate degradation mechanisms, optimum conditions for enzymatic activity and the regulation of enzyme by inhibiters (Sathyanarayana and Panda 2003).

Of the many microorganisms *Bacillus spp*. are known to produce variety of extracellular enzymes and they have a wide range of industrial applications (Annamalai et al., 2011; Amin et al., 2017). The advantage of using micro-organisms for the production of enzymes is that these are not influenced by climatic and seasonal factors, and can be subjected to genetic and environmental manipulations to increase the yield (Zeni et al., 2020)

The microbial pectinase production varies according to the composition of growth medium and the cultivation conditions i.e., pH, temp, aeration, agitation and incubation time (Thakur et al., 2010). Carbon sources also effect the production of pectinase. As observed PGA, lactose, pectin increased pectinase production (Kashyap et al., 2003). In *Aspergillus fumigatus* sucrose yielded maximum pectinase production (Phutela et al., 2005). Among the nitrogen sources for pectinase production the maximum yield of pectinase was shown by yeast extract pectinase production is inhibited by glycine, urea, ammonium nitrate while wheat bran, peptone, ammonium chloride, yeast extract enhance pectinase production (Colla et al., 2017). The optimum pH for growth and pectinase production for most of the bacteria is 7-10 (Ahlawat et al., 2009; Tepe and Dursun 2014). Some bacterial strains; *Streptomyces* QG-11-3 and *Aspergillus aculeatus* produced PGase active at pH 3.0 (Torres et al., 2006).

The activity of pectinases depends on thermal stability. Pectinase production by *Bacillus subtilis* the optimum temperature was found to be 37°C (Ahlawat et al., 2009). PGase from fungi have optimum activity at 50°C while from yeast the temperature varies from 40°C to 60°C (Torres et al., 2006). The activity of Endo PGase is reduced due to Cu^{2+} and Hg^{2+}. Metal ions Hg^{2+}, Zn^{2+}, Mg^{2+} inhibit enzyme production due to inhibition by thiol group blocking agents as there is possible involvement of the thiol group in the enzyme s active site (Ahlawat et al., 2009; Suneetha and Khan 2011). Mn^{2+} increases the PGase activity; however Li^{2+}, Fe^{2+}, Rb^{2+} have no effect on the activity.

The time of fermentation i.e. incubation period of organisms had a profound effect on microbial product formation (Bennamoun et al., 2016; Murad and Foda 1992; Murad and Salem 2001). Reda et al. (2008) found that the level of polygalacturonase increased gradually with increasing the incubation period up to a maximum of 96 h by *Bacillus firmus*-1-10104 under solid state fermentation conditions. The effects of various organic and inorganic nitrogen sources on the pectinase production were extensively studied. The observations of Hours suggested that lower levels of $(NH_4)2So_4$ (0.16%), or K_2HPO_4 (0.1%) added to the growth medium as inorganic nitrogen sources did not influence pectinase yield (Hours et al., 1988). Reda found that the maximum value of polygalacturonase productivity by *Bacillus firmus*-1-10104 reached up to 350 U mL^{-1} in the presence of peptone as a nitrogen source in the growth medium. In addition to this organic nitrogen sources showed higher endo, exo pectinases activities than inorganic nitrogen sources (Vivek et al., 2010).

Optimum growth affecting the growth of organism and its metabolism can be achieved by an adequate supply of carbon as energy source. Reda et al. (2008) reported that *Solanum tuberosum* (ST) peels was the best carbon source for polygalacturonase production by *Bacillus firmus*-1-10104 under solid state condition. *B. megaterium* enzymes and products have been used in industrial applications for several years and are also effective in immobilized systems, making industrial processes even more efficient. An extensive review of B. megaterium emphasizing its commercial applications has been published (Vary, 1992). In the present study, an efficient exo-polygalacturonase producing strain was isolated from potent sites of pectin waste and identified as *Bacillus megaterium* followed by optimizing favourable conditions for growth and exo-polygalacturonase production.

MATERIAL AND METHODS

Cultures were selectively isolated from untreated non-rhizospheric potent soil mainly from pectin producing industrial wastes initially by serial dilution and plating technique using pectin enrichment method. Sample was inoculated into Nutrient Agar medium and incubated for 24 to 48 hours at 37 °C and isolates were subjected for their pectinolytic property by puncturing the medium on Citrus pectin agar (CPA) medium which is a quantitative test for pectin degrading bacteria. Potential isolates were achieved at mesophilic temperatures ranging from 30 to 37 °C. All morphological colonies were purified by repeated streaking.The medium was the same used for isolation of cultures, supplemented with 2% agar agar. Pure cultures were inoculated by puncture in the medium and incubated for 48h at 30°C. After the colonies reached around 3 mm, iodine –potassium iodide solution (1.0 gm Iodine, 5g of KI and 330 ml H_2O) was added to detect clearance zones (Zeni et al., 2020).

The potent pectinolytic isolates were also checked for cellulolytic, proteolytic and amylolytic activities by using carboxymethylcellulose (CMC) agar medium, casein agar medium and starch agar medium through getting clearing zones. Strains presenting large clearing zones were used for enzyme production on liquid medium same used for screening. Cultures were grown in 150 mL Erlenmeyers flasks with 100 ml of medium in rotary shaker of 150 rpm at 30 °C. After 48 hours the biomass was separated by centrifugation at 10000xg for 20 minutes and the supernatant was used to evaluate polygalacturonase (PG) activity. The supernatant was used for further enzymatic activity studies (Soares et al., 2001).

Exo polygalacturonase activity was determined by measuring the release of reducing groups using the dinitro salicyclic acid reagent (DNS) assay by Miller (1959). The reaction mixture containing 0.8 ml of 1% citrus pectin with 67% metoxilation in 0.2 M citrate phosphate buffer, pH 6.0 buffers and 0.2 ml of culture supernatant, was incubated at 37 °C for 10 min. One unit of enzymatic activity (U) was defined as 1 µmol of galacturonic acid released per minute. Citrus Pectin medium (100 ml in 500 ml Erlenmeyer flasks) was inoculated with 1 ml overnight culture of *Bacillus megaterium* and incubated at 37 °C with vigorous aeration in a shaker at 150 rpm for 5 days. Cells were separated by centrifugation at 8000xg for 20 minutes at 4 °C. The cell free culture (supernatant) was used as crude enzyme source. All steps of purification were carried out at 4 °C. The enzyme in the crude preparation was precipitated by the addition of Ammonium sulphate (30-90% saturation) and kept overnight at 4 °C and centrifuged in a refrigerated centrifuge (Remi C24) at 10000g for 30 min. The precipitate was removed and supernatant was again subjected with further addition of ammonium sulphate in view to remove other proteinaceous material.

The resultant precipitate bearing high pectinase activity was dissolved in the small quantity of 0.01M Tris-HCl buffer (pH 6.0) and dialyzed (in dialysis bag, Sigma 10-12 kDa) overnight against the same buffer with constant stirring and was subjected to further purification. During the course of dialysis, the buffer was frequently changed with the fresh lot until no traces of ammonium were found upon testing with Nessler's reagent (Afifi et al., 2002). Production medium was supplemented with different nitrogen sources at an equimolar of nitrogen that present in diammonium sulphate in pectin medium. The applied nitrogen sources were ammonium molybdate, ammonium chloride, ammonium oxalate, ammonium nitrate, diammonium hydrogen phosphate, potassium nitrate, gelatin, peptone, casein and urea. Different carbon sources were introduced into the pectin medium at an equimolecular amount located at 1% (W/V) glucose. The carbon sources were represented by glucose, sucrose, starch, lactose, maltose, galactose, fructose, cellulose and pectin.

The optimum pH was determined with tri sodium citrate 1% (w/v) as the substrate dissolved in different buffers (Tris-Hcl 3.0, Citrate phosphate, pH 5.0, sodium phosphate 7.0, glycine-NaOH pH 9). The pH was adjusted at different pH values 2, 4, 6, 8, 10, 12 by using sodium citrate buffer. The effect of pH was determined by pre-incubating enzyme and substrate at different pH values (2-12) for 24 hours at room temperature. The effect of temperature on enzyme activity was determined by performing standard assay procedure within a temperature range from 0 to 75 °C for 24 hours.

The effect of different metal ions on pectinase activity was determined by the addition of the corresponding ion at a final concentration of 0.1 mM and the reaction mixture was assayed under standard conditions. The enzyme was carried out in the presence of CaCl2, $MgSO_4$, $CuSO_4$ and inhibitor like EDTA. The effect of surfactants on exo-PG was determined by using the corresponding ions at a concentration of 0.1 mM and the reaction mixture was assayed under standard conditions. The enzyme was carried out in the presence of surfactants like Tween 80, Tween 20 and Triton X-100.Different concentrations of citrus pectin (0.5%, 1%, 1.5% and 2%) were applied to determine the effect of substrate concentration on exo-PG activity. Exo-PG enzyme activity was estimated for different time intervals (5-30 minutes) at 55 °C. Enzyme stability was measured at different temperatures (35 to 85 °C) for one hour.

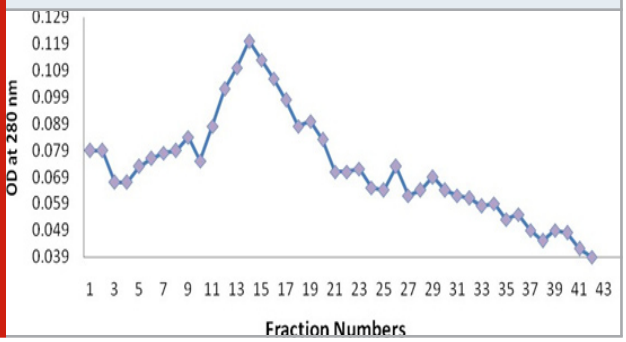
RESULTS AND DISCUSSION

Based on the above experiments it is clearly evidenced that *B. megaterium* was showing maximum activity. Exo- PG (EC 3.2.1.67) is an essential component of pectinase complex and is the key enzyme used by the bacteria to decompose pectin. Exo-PG randomly hydrolyzed internal glyosidic linkages, which result in rapid decrease in polymer length and a gradual increase in the reducing sugar concentration. In view of its importance in saccharification process of pectic substrates and also poor production in some bacterial strains, its search in other organisms attracts a great deal of attention. Therefore, production and purification of exo-PG by *B. megaterium* isolated in this study was emphasized. The cell free culture filtrate obtained by orbital shaker in 100 mM Tris-HCl was used as crude enzyme source. In the culture filtrate a total activity of 0.98 U and total protein content of 2.35 mg with specific activity of 0.41 U/mg, with recovery 100%.

The supernatant was fractionated by precipitation with ammonium sulphate at 30-90% saturation. The precipitate was suspended in 0.1 M Tris-HCl buffer (pH 6.0) and dialyzed against same buffer with constant stirring. After dialysis protein content was determined. The purified fraction from ammonium sulphate has a total activity of 0.6 U, with total protein content of 0.68 mg with specific activity of 0.882 U/mg with 67.4% recovery with purification fold of 2.578. Further the fraction from ammonium sulphate precipitation was subjected to ion exchange chromatography by means of DEAE cellulose CL21 column. Fraction was obtained showing a total activity of 0.80 U and total protein with 0.82 mg. The specific activity calculated was 0.97 U/mg with recovery

of 81% and purification fold of 2.3 (Figure 1).

Figure 1: Fractions from Sephadex G100 column



The fraction from DEAE cellulose CL 21 chromatography was further subjected to gel filtration using Sephadex G100 column. This fraction had total activity of 0.63 U with a recorded total protein content of 0.174 mg with specific activity of 3.6 U/mg. The enzyme was recovered with a purification fold of 8.8 and 64% recovered. The fraction from Sephadex G100 column was subjected to SDS- PAGE to determine purity of enzyme (Table 1).

The influence of different physiological factors like pH, temperature, metal ions, carbon sources, nitrogen sources, surfactants, substrate concentration, time and temperature stability was observed on exo-PG activity. Influence of pH on exo-PG activity by bacterial isolates was examined. Individual enzyme components of exo-PG had shown maximum activity at pH 6 to 8 in both isolates. As the isolate was found active at pH 7, it can be used in degradation of organic matter, detergents and sewage treatment (Table 2). High activities of exo-PG (0.96 U/ml) produced at pH 6.0 to 8.0. Above and below the pH range of 6-8, a decrease in the growth was observed.

Table 1. Purification of exo-Polygalacturonase

Purification Step	Total Protein (mg)	Activity (U)	Specific Activity (U/mg)	Yield (100%)	Purification Fold
Crude Enzyme	2.35	0.98	0.41	100	1
Ammonium Sulphate precipitation	0.68	0.6	0.882	67.4	2.578
Dialysis	0.82	0.8	0.97	81	2.3
Sephadex G – 100	0.174	0.63	3.6	64	8.8

Table 2. Effect of pH on exo-PG activity of *B. megaterium*

	pH	N	Mean	Std. Deviation	F-value	P-value
	2	3	0.3333 a	0.01528		
	4	3	0.4200 b	0.01000		
	6	3	0.9633 c	0.01528		
B. megaterium	8	3	0.9500 c	0.02000	971.242**	0.000
	10	3	0.5333 d	0.01528		
	12	3	0.4433 b	0.01528		
	Total	18	0.6072	0.26155		

** Significant at 1% level; Note: The same letter indicates insignificant difference in any pair of media pH according to Duncan's Multiple Range Test (DMRT).

Results were noted in Table 2. From the F-value one can understand that there is significant difference among the impact of pH on the exo-PG activity in both organisms. Maximum activity was observed at pH 6 and 8 in both isolates. Further DMRT suggest that the same level of growth was observed at pH 6 and 8 of *B. megaterium* and then followed by pH 4 and 12. Temperature highly influences the metabolic reactions through enzymatic activities, thereby affecting the growth of the organism. Effect of temperatures on enzyme production indicated that maximum exo-PG enzyme was shown at 55oC. The growth, extracellular protein, total soluble sugar content and exo-PG enzymes were assayed at 72 hr of incubation (Table 3). This culture filtrate also yielded higher activity in terms of exo-PG with 0.97 U/ml in *B. megaterium*. Exo-PG activity was showing significant difference at 1% level among the temperatures. Since p - value 0.000< 0.01 for the corresponding F- value (376.167**). At 55 °C *B. megaterium* show maximum activity 0.97 u/ml, respectively. This temperature is most useful for the

degradation purposes of organic matter when compare to fungal pectinase.

various temperatures.Effect of different metal ions and inhibitors on exo-PG production in *B. megaterium* was studied. Metal ions tested had shown stimulatory as well as inhibitory effect on microorganisms. We found that $CaCl_2$ showed increased exo-PG activity when compare to other metal ions. This culture filtrate also yielded higher activity in terms of exo-PG with 0.25 U/ml. Inhibitors like EDTA, NaN3 at 1mM concentration inhibited the activity i.e., (0.07U/ml) **. One way ANOVA results revealed that metal ions and inhibitors play an important role on exo-PG activity (Table 4). $CaCl_2$ has shown stimulatory effect on exo-PG activity. Inhibitors like EDTA and NaN_3 were also greatly influencing the exo PG activity.

Table 3. Effect of Temperature on exo pg activity by *B. megaterium*

	Temperature	N	Mean	Std. Deviation	F-Value	P-Value
	25	3	0.3467 a	0.02517		
	35	3	0.4167 b	0.01528		
	45	3	0.4933 c	0.01528		
B. megaterium	55	3	0.9700 c	0.02000	376.167**	0.000
	65	3	0.4400 d	0.02646		
	75	3	0.3700 b	0.02000		
	Total	18	0.5061	0.21966		

** Significant at 1% level; Note: The same letter indicates insignificant difference among the

Table 4. Effect of metal ions on exo-PG activity by *B. megaterium*

	Metal ion	N	Mean	Std. Deviation	F-value	P-value
	$CuSO_4$	3	0.1933 a	0.01528		
	$MnSO_4$	3	0.2233 b	0.02082		
B. megaterium	$CaCl_2$	3	0.2500 b	0.01000	85.690**	0.000
	EDTA	3	0.0700 c	0.02000		
	NaN3	3	0.0567 c	0.01528		
	Total	15	0.1587	0.08400		

** Significant at 1% level

Exo-PG production and secretion of extracellular protein on citrus pectin medium with different carbon sources at 1% level was compared for the organism. Results of this comparative study are represented in Table 5. Of all the carbon sources tested in this study, Lactose and Citrus Pectin elicited the production of highest titres of exo-PG activity with 1.5 U/ml in *B. megaterium*. Lactose was the best carbon source among all the carbon sources.

Nitrogen is one of the important elements required for growth of microorganisms. Provision of utilizable form of nitrogen source to organisms is the basic requirement to be fulfilled for optimal growth. In order to find out the best utilizable form of nitrogen source for growth, extracellular protein and pectinase production by bacteria on citrus pectin medium supplemented with different nitrogen source such as ammonium sulphate, ammonium molybdate, ammonium chloride, ammonium oxalate, ammonium nitrate, K_2HPO_4, KNO_3, gelatin, peptone, casein, and urea were determined. The results obtained from this experiment were presented in table and graph. Peptone followed by casein and K_2HPO_4 were the best nitrogen sources for *B. megaterium*. The highest activity of exo-PG produced was 0.66 U/ml (Table 6). The growth of the both isolates showing a significant difference at 1% level among the nitrogen sources. Since, p value 0.000<0.01 for the corresponding F–value is 294.221. The results suggested that the difference between the same alphabets was insignificant.

Influence of surfactants such as Triton-X-100, Tween-80, and Tween-20 on exo-PG production by *B. megaterium* was determined in the same manner. Exo-PG production on the medium amended with surfactants was showed in Table 13 and Figure 14. The medium with Triton X-100 had yielded more extracellular protein and highest

pectinolytic activity of 0.37 U/ml (Table 7). Since p –value 0.000 <0.01 for the corresponding F–Value 141.23. Triton X-100 showed maximum exo-PG activity.

Substrate concentration also plays an important role on the exo pg activity. CP medium was amended with 0.5%, 1%, 1.5%, 2%, 2.5% etc and checked for the better activity. 1% citrus pectin shows maximum activity on exo-PG compare to other concentrations. One way ANOVA reveals that there is significant substrate concentration on exo-PG activity. One percent citrus pectin shows maximum activity on exo-PG activity 0.73 U/ml. Since p-value 0.000<0.01 for the corresponding F-value is 138.29**.

Table 5. Effect of different carbon sources on exo-PG production

	Carbon sources	N	Mean	Std. Deviation	F-value	P-value
	Lactose	3	1.5367 a	0.01528		
	Sucrose	3	0.3600 b	0.01000		
	Starch	3	0.9467 c	0.01528	1576.858**	0.000
	Glucose	3	0.4367 d	0.02082		
B. megaterium	Maltose	3	0.5633 e	0.01528		
	Galactose	3	0.7400 f	0.02000		
	Fructose	3	0.5600 e	0.01000		
	Cellulose	3	0.4333 d	0.01528		
	Pectin	3	0.9667 c	0.02082		
	Total	27	0.7270	0.35996		

** Significant at 1% level; **Note:** The same letter indicates insignificant difference among the various carbon sources.

Table 6. Effect of nitrogen sources on exo-PG activity by *B. megaterium*

	Nitrogen Sources	N	Mean	Std. Deviation	F-Value	P-Value
	Ammonium	3	0.2333 a	0.02082		
	Sulphate					
	Ammonium	3	0.1767 b	0.01528		
	molybdate					
B. megaterium	Ammonium	3	0.2567 c	0.01528	294.221*	0.000
	chloride					
	Ammonium	3	0.2333 a	0.01528		
	oxalate					
	Ammonium	3	0.3133 d	0.01528		
	nitrate					
	K2HPO4	3	0.5633 e	0.01528		
	KNO3	3	0.4867 f	0.01528		
	Gelatin	3	0.4367 g	0.02082		
	Peptone	3	0.6667 h	0.01528		
	Casein	3	0.5733 e	.01528		
	Urea	3	0.2767 c	.02082		
	Total	33	0.3833	.16330		

** Significant at 1% level; Note: The same letter indicates insignificant difference among the various nitrogen sources.

Stability of exo-PG was studied for one hour at 65^{0}C temperature. One way ANOVA was carried out whether stability of the exo-PG was significant. At 65 °C temperature the enzyme stability was for one hour was 0.73 U/ml. Based on DMRT analysis the influence of different temperatures for one hour was significant. The alphabets showed insignificant among the temperatures for one hour.

The activity of each enzyme component is greatly influenced by factors like pH, temperature, metal ions / inhibitors, surfactants, substrate concentrations, time,

temperature stability and nutrients which can be best monitored for the maximum activity of the enzyme complex thus the degradation. Pectinolytic enzymes or pectinases are a heterogeneous group of enzymes that hydrolyze the pectic substances present in plants. They include polygalacturonase, pectin lyase, and pectin methyl esterase that hydrolyze the glycosidic bonds of pectic substances. Endo polygalacturonase (EC 3.2.1.15) and exo polygalacturonase (EC 3.2.1.67) are the enzymes of particular interest to industry because they act on pectin, hydrolyzing its internal and external glycosidic bonds, producing shorter pectin molecular structures, decreasing the viscosity, increasing the yield of juices, and determining the crystalline structure of the final product. Some of the bacterial species producing pectinases are *Agrobacterium tumefaciens, Bacteroides thetaiotamicron, Ralstonia solanacearum,* and *Bacillus sp* (Bennamoun et al., 2016; Murad and Foda 1992). After identification of bacterial cultures, exo polygalacturonase production was done in citrus pectin agar (CPA) medium. Pectinolytic activity of bacterial cultures was determined according to dinitro salicillic acid by Miller (1959) and Colla et al (2017).

Table 7. Effect of surfactants on exo-PG activity by *B. megaterium*

	Surfactants	N	Mean	Std. Deviation	F-value	p-value
	Tween80	3	0.1900 a	0.01000		
B. megaterium	Tween20	3	0.2433 b	0.01528	141.235**	0.000
	Triton X-100	3	0.3733 c	0.01528		
	Total	9	0.2689	0.08253		

** Significant at 1% level

Table 8. Effect of substrate concentration on exo-PG activity by *B. megaterium*

	Substrate Conc.	N	Mean	Std. Deviation	F-value	p-value
	0.50%	3	0.4733 a	0.01528		
	1%	3	0.7333 b	0.01528		
B. megaterium	1.50%	3	0.6267 c	0.01528	138.293**	0.000
	2%	3	0.5267 d	0.01528		
	2.50%	3	0.4733 a	0.02082		
	Total	15	0.5667	0.10486		

** Significant at 1% level; Note: The same letter indicates insignificant difference among the various concentrations of substrate.

Individual enzyme components of exo PG secreted into the culture medium of *B. megaterium* was estimated in accordance with methods listed by (Amin et al., 2017). The PGase activity was assayed by estimating the amount of reducing sugar released under assay conditions. The reducing sugar produced in the reaction mixture was determined by dinitro salicylic acid (DNS) method. One unit of exo polygalactoronic acid activity was defined as the amount of enzyme releasing 1 µ mole of galactuornic acid per minute. The extracellular exo polygalacturonase enzyme of *B. megaterium* sps, was purified 8.8 fold with an yield of 31.4%. Its specific activity was 3.6 U/mg. The Physiological conditions play a vital role in enzyme production (Zeni et al 2020).

These conditions include environmental factors such as temperature, pH, substrate concentration, metal ions, carbon sources, nitrogen sources, surfactants and stability of exo-polygalacturonase which have shown significant effect on exo-PG activity of bacterial isolates (Tepe and Dursun 2014). The purified enzyme exhibited optimum polygalacturonase activity at initial pH of the production medium was adjusted to 6-8 (Table 2) and there was a drastic decrease (80%) in enzyme activity at above or below pH range of 6-8. A pH range of 6–8 has been reported for maximum PGase production of *B. megaterium*. High activities of exo-pg (0.96 U/ml) production were observed at pH 6.0 to 8.0.

Most favourable production temperature for PGase production was found to be 55 °C (Table 3). *B. megaterium* produced maximum PGase activity when incubated at 55 °C for 72 hours. Further effect of different metal ions and inhibitors on exoPG production was studied. Metal

ions tested had shown stimulatory as well as inhibitory effect on microorganisms. Stimulatory effect was shown by $CaCl_2$ which increased exo-pg activity when compare to other metal ions. Inhibitors like EDTA, NaN3 at 1mM concentration inhibited the activity in both the organisms (Table 4). This culture filtrate also yielded higher activity in terms of exo pg with 0.21 U/ml and 0.25 U/ml respectively. *B. megaterium* pectinolytic potential was further assessed by determining the effects of nutrients on exo-pg production. These nutrients include Carbon sources, Nitrogen sources, surfactants, substrate concentration etc. of all the carbon sources tested in this study, Lactose and Citrus Pectin elicited the production of highest titres of exo-PG activity with 1.5 U/ml of B. Megaterium (Table 5).Nitrogen is one of the important elements required for growth of microorganisms. Peptone followed by casein and K_2HPO_4 were the best nitrogen sources for *B. megaterium*. The highest activity of exo polygalacturonase with peptone was 0.66 U/ml by *Bacillus megaterium* (Table 6). Influence of surfactants such as Triton-X-100, Tween-80, and Tween-20 on exo polygalacturonase production by *B. megaterium* was also determined in the same manner. The medium with Triton x-100 had yielded more extracellular protein and highest pectinolytic activity of 0.25U/ml and 0.37 U/ml respectively (Table 7).

Substrate concentration also plays an important role on the exo pg activity. CP medium was amended with 0.5%, 1%, 1.5%, 2%, 2.5% etc and checked for the better activity. 1% citrus pectin shows maximum activity on exo-PG compare to other concentrations (Table 8). Stability of exo PG was also studied for one hour. At 65oC temperature the enzyme stability was for one hour was 0.73 U/ml. *B. megaterium* shows best activity at 65oC for one hour.The maximum activity of polygalacturonase from *B. megaterium* was obtained using 0.5 ml of enzyme after 15 minutes' incubation at 50 °C and pH of 5.0 (Table 9). The activity of each enzyme component is greatly influenced by factors like pH, temperature, metal ions / inhibitors, surfactants, substrate concentrations, time, temperature stability and nutrients which can be best monitored for the maximum activity of the polygalacturonase production.

Table 9. Temperature Stability of exo-PG by *B. megaterium*

	Temp. Stability	N	Mean	Std. Deviation	F-Value	P-Value
	35	3	0.4300 a	0.02000		
	45	3	0.5100 b	0.02646		
	55	3	0.6700 c	0.02000		
B. megaterium	65	3	0.7367 d	0.01528	284.015**	0.000
	75	3	0.3067 e	0.01528		
	85	3	0.2833 f	0.01528		
	Total	18	0.4894	0.17575		

** Significant at 1% level; Note: The same letter indicates insignificant difference among the various temperature stabilities.

CONCLUSION

Enzyme production is one of the broad areas of biotechnology which accounts for about 1.5 billion of the world market. The enzymes from microbial origin were found to be more advantageous than others. Pectinase accounts for 10% of global industrial enzymes produced and their market is increasing day by day. Pectinolytic enzymes are produced by many organisms like bacteria, fungi, yeasts, insects, nematodes, protozoan and plants. In the present study production of exo polygalacturonase using mango fruit waste was carried out and the production conditions optimized. Several microorganisms are capable of using these substances as carbon and energy sources by producing a vast array of enzymes in different environmental conditions.

Serial diluted samples of fruit waste were screened for exo-PG produced by bacteria. Out of the 16 bacteria grown on screening medium (citrus pectin agar medium) only 6 strains showed clear zones. Among the six isolates, 1 strain showed maximum clearing zones and found to be pectinase producers. These strains were preliminarily identified. Then it was identified as *Bacillus megaterium* by IMTECH, Chandigarh. Enzyme activity was measured and protein estimation was done for the exo PG activity. Optimum conditions for the exo PG production was studied pH 6-8, temp-55 °C, metal ions-$CaCl_2$, carbon sources-lactose and pectin, nitrogen sources peptone and casein surfactants like Triton X-100, substrate concentration 1%, effect of time 15 to 20 minutes, stability of the enzyme 65 °C for 1 hour.

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