

Analysis of Extra-Cellular Productions of *Bacillus subtilis* Sub-Merged Fermentation Cultures Supplemented with Rotten Potatoes and Sugar Beets

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ABSTRACT

The *Bacillus subtilis* (k1) is the SAFE microorganism with enormous extracellular productions and it has ability to grow under large versatile nutrient conditions. Among the agriculture wastes, the rotten potatoes and sugar beets are being carbon-rich natural renewable agro-wastes. In compliance with current study, *B. subtilis* was fermented for 18 hrs on LB growth medium supplemented with 12.5 % extracts (v/v) of rotten potatoes and sugar beets i.e. LB₀ [1 % Bacto-trypton, 0.5 % NaCl, 0.5 % yeast extract in dH₂O, LB₁ (1/8 LB₀), LB₂ (LB₁, peels of potatoes), LB_{2a} (LB₁, peeled potatoes), LB₃ (LB₁, peels of sugar beet) and LB_{3a} (LB₁, peeled sugar beet). The significantly higher cell growth rate was observed in LB₃ and LB_{3a} cultures. Maximum reducing sugars observed in LB_{2a} while fructose contents in LB₃ (3.214±0.077 mg ml⁻¹) and LB_{3a} (2.971±0.044 mg ml⁻¹) cultures. Simultaneously, higher enzymatic activities (p ≥ 0.05) showed by amylases on LB_{2a} and LB_{3a}, while *xylanases* on LB_{2a} and *pectinases* on LB₃ cultures. For instance, pectinases activities remarkably exceeded at 50°C in LB₃ cultures maintained for 30 min. Overall, *B. subtilis* (k1) can grow on both rotten potatoes and sugar beets with hug numbers extracellular productions, while sugar beets based cultures gave the best returns in bacterial sub-merged fermentation for the industrial enzymes productions.

KEY WORDS: *Bacillus subtilis*, POTATOES, SUGAR BEETS, GLUCOSE, ANTIOXIDANTS, FREE PROLINES, PECTINASE, PHYSICAL CONDITIONS.

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INTRODUCTION

The agriculture wastes are the unwanted materials produced from agro-based activities. It has been used as feed for the animals and fish. Even many are unsuitable for direct animal consumption so they are treated mechanically and chemically for their conversion into edible form (Jayathilakan et al., 2012). These costless waste materials are aggregated from the agriculture land to kitchen during its processing. With passage of time, these are converted to rotten form before its consumption or to get its end product (Obi et al., 2016). It depends on the rate of production to its consumption over the time frame. The potato and sugar beets are the crops, which are observed into rotten form in the vegetable markets including the wastes excised during their processings (Wadhwa and Bakshi, 2013). The agriculture waste are comprised on mainly cellulose, hemicelluloses and lignin chemically. Utilization of these renewable carbon resources depends on their degradation methods (Yahya et al., 2015; Emadian et al., 2017).

The natural degradation of agricultural wastes in the soil has been remained effective for the control of soil erosion and revegetation (Anastasi et al., 2005) and it is slower process. The chemo-mechanical degradation of the agriculture wastes is expensive and could be hazardous for human health, while biodegradation is cheap and manageable method. The soil born organisms are composting the organic wastes into organic fertilizers of soil through biological processes (Gautam et al., 2010). It means that a variety of microorganisms are able to grow on the agriculture wastes including *Bacillus subtilis* (An et al., 2018).

The agriculture wastes of crops contain sucrose (sugarcane, sugar beets), starch materials (corn, potatoes) and lignocellulosic materials (wood, grasses) are renewable lignocellulosic wastes available at lowest costs (Jenkins and Alles, 2011). However, their accumulation is causing various environmental problems, while these are potentially valuable sources to produce many value-added products like as fructose, glucose, ethanol, organic acids, food additives and enzymes (Pandey et al., 2016). Meanwhile, the type of production depends on the chemical composition of lignocellulosic source and the applied fermentation microorganism (Salazar et al., 2016; Tramontina et al., 2020).

The value added-renewable lignocellulosic agro-industrial waste can be used as cellular growth energy source and other secondary metabolites productions (Rocha et al., 2014). Meanwhile, efficient bio-tools for the management of agro-wastes are the microbial enzymes. The microorganisms are producing a variety of cellulases, proteinases and pectinolytic enzymes (Jayani et al., 2010; Tripathi et al., 2014). These are significantly eco-friendly industrial enzymes (Singh et al., 2009). Especially *Bacillus subtilis* are potential fermentation microorganism have been using in the global food, textile industries to composting processes (Nawawi et al., 2017).

Table 1. Composition of different bacterial nutrient media used for *Bacillus subtilis* (k1) growth supplemented with potatoes and sugar beets as fermentation substrate

#s.	Medium	Composition of medium
01	LB ₀	1 % Bacto-trypton, 0.5 % NaCl, 0.5 % yeast extract in dH ₂ O (w/v)
02	LB ₁	1/8LB ₀ in dH ₂ O (v/v)
03	LB ₂	LB ₁ +25 ml peels of potatoes(25 %, v/v)
04	LB _{2,a}	LB ₁ +25ml peeled potatoes (25 %, v/v)
05	LB ₃	LB ₁ +25 ml peels of sweet-potatoes (25 %, v/v)
06	LB _{3,a}	LB ₁ +25 ml peeled sweet-potatoes (25 %, v/v)

Note: The final concentration of each extract adjusted to 12.5 %. Individual culture maintained in 4 replicates and volume of each replicate maintained 50 ml before its sterilization.

Figure 1: The agro-wastes used for *Bacillus subtilis* (k1) growth as carbon source in sub-merged fermentation cultures [rotten potatoes (a) and sugar beets (b)]



Perhaps the higher enzyme production costs are the major constraint for their commercialization. Though, selection of cheap carbon source, high yielding bacterial strains and optimal fermentation conditions significantly can reduce the enzyme production costs (Liu and Kokare, 2017; Tramontina et al., 2020). In present study is aimed to analyse the net production of reducing sugars especially fructose, glucose and extracellular enzymes. These productions with *Bacillus subtilis* might be helpful for the determination of its abilities and efficiencies for the saccharification of lignocellulosic rich agriculture wastes of rotten potatoes and sugar beets. This work could be useful for the production of stable commercial organic compounds and industrial enzymes able to withstand against the hazardous industrial conditions.

MATERIAL AND METHODS

Preparation of inoculum: The inoculum of *Bacillus subtilis* (k₁) prepared from its glycerol stock. For that it is mobilized in 2 ml LB₀ (TY) medium (Table 1), which was incubated at 37°C with constant shaking speed 250 rpm for overnight (De Vries et al., 2004). The 100 µl of above

B. subtilis culture sub-cultured in 5 ml LB₀ medium. It was again incubated under same physical circumstances for 30 minutes (Table 1). Now this master culture is inoculated in the agro-based waste fermentation medium (Table 1) with final OD600 upto 0.02

Preparation of sugar beets and potatoes based agro-fermentation culture: The sugar beets and potatoes based fermentation cultures heightened in liquid nutrient LB₀

medium (5 g l⁻¹ yeast extract, 5 g l⁻¹ NaCl, 10 g l⁻¹ Bactotrypton, pH 7.0). Exact 12.5 % potatoes and sugar beets extracts (peels and peeled-potatoes and sugar beets) sustained in 1/8 strength of LB₀ medium, while the LB₀ medium itself was considered as standard positive control for the growth of fermentation organism as well as 1/8 LB₀ medium as essential nutrient deficit control medium (Table 1). All of these cultures sterilized at 121°C for 20 minutes than cool down before inoculation at the room temperature.

Table 2. Comparative biocomponents and enzyme productions in various nutrient cultures of *Bacillus subtilis* (k.) supplemented with potatoes and sugar beets as carbon sources

#s.	Characters/ Parameters	LB ₀	LB ₁ (1/8LB ₀)	LB ₂	LB _{2a}	LB ₃	LB _{3a}	Data significance
01.	Total sugars (mg ml ⁻¹)	^{cd} 3.664±0.045	^d 1.832±0.034	^{bc} 3.847±0.055	^c 3.752±0.036	^a 4.188±0.045	^b 3.949±0.051	***
02.	Reducing sugars (mg ml ⁻¹)	^c 2.387±0.050	^e 1.392±0.077	^{bc} 2.831±0.058	^d 1.758±0.048	^a 3.214±0.077	^b 2.971±0.044	***
03.	Total proteins (mg ml ⁻¹)	^b 8.743±0.033	^e 1.799±0.056	^c 8.221±0.054	^d 7.792±0.052	^a 9.684±0.103	^{bc} 8.630±0.028	***
04.	Fructose (mg ml ⁻¹)	^{de} 0.722±0.003	^e 0.147±0.004	^a 1.078±0.003	^d 0.789±0.003	^{ab} 1.058±0.002	^c 1.011±0.002	***
05.	Citric acid (mg ml ⁻¹)	^{ab} 0.086±0.003	^{cd} 0.050±0.003	^{abc} 0.087±0.004	^a 0.091±0.002	^{bcd} 0.063±0.003	^{de} 0.049±0.003	**
06.	Ascorbic acid (mg ml ⁻¹)	^d 9.44±0.108	^f 3.030±0.105	^c 12.49±0.429	^{de} 9.075±0.108	^a 14.67±0.114	^b 13.28±0.130	***
07.	Proline (mg ml ⁻¹)	^{cd} 1.007±0.006	^e 0.639±0.005	^{ab} 1.161±0.011	^a 1.338±0.006	^{bc} 1.122±0.005	^{ab} 1.260±0.004	***
08.	Glycinebetaine (mg ml ⁻¹)	^{cde} 0.131±0.003	^f 0.087±0.004	^{def} 0.129±0.002	^{cd} 0.140±0.005	^{bc} 0.169±0.003	^a 0.198±0.004	**
09.	Phenolics (mg ml ⁻¹)	^{ab} 7.264±0.037	^e 1.892±0.040	^a 7.450±0.022	^{bc} 6.403±0.062	^b 6.669±0.092	^{cd} 6.081±0.023	***
10.	Flavonoids (mg ml ⁻¹)	^{bc} 1.862±0.060	^{cde} 1.142±0.025	^{def} 1.273±0.013	^{bcd} 1.387±0.032	^a 4.799±0.037	^b 4.451±0.040	***
11.	Antioxidants (mg ml ⁻¹)	^a 0.865±0.013	^d 0.242±0.002	^{ab} 0.671±0.008	^{bcd} 0.575±0.005	^a 0.673±0.003	^{bc} 0.591±0.006	***
12.	Phosphates (mg ml ⁻¹)	^a 0.984±0.003	^d 0.345±0.007	^{ab} 0.964±0.005	^{ab} 0.967±0.004	^{abc} 0.958±0.006	^{bcd} 0.939±0.002	**
13.	Methionine (mg ml ⁻¹)	^c 0.181±0.005	^e 0.090±0.002	^{cd} 0.123±0.003	^{de} 0.115±0.006	^b 0.223±0.003	^a 0.240±0.004	***

Note: Each parameter is presented with mean values of 4-replicates with their standard error and significances at 0.05 level (5%) with a,b,c,d ... letters for DMR test.

Preparation of agro-fermentation substrate: The old and rotten potatoes and sugar beets collected from the nearby local vegetable market (Fig 1). These rotten potatoes and sugar beets were washed with running tap-water to clean dust or soil propely. Their skin or peels were separated with fine knife than peels and peeled stuff of both potatoes and sugar beets weighted exactly 50 g. These weighed material crushed with grinder grinder in 50 ml sterilized dH₂O (w/v). After that the grinded mixture centrifuged at 4,000 rpm at room temperature for 10 min. The supernatant preserved at 4°C for next

use in the agro-fermentation medium preparation while its pallet was discarded .

Harvesting of Bacillus fermentation culture: The inoculated cultures incubated for 18 hours at 37°C with constant shaking at 250 rpm. After 18 hrs of incubation of the fermentation cultures, they were harvested. Before the collection of culture for harvest, their OD600 was measured. These cultures were centrifuged for 10 minutes at 7,000 rpm. The supernatants of the cultures transferred to the clean dark-colored glass-bottles, while

its pallet was discarded. The supernatant of each culture stored at 4°C for next it was used as a sample for the measurements of different biochemical and activities of enzyme outcome.

Figure 2: Comparative cell growth rates of *Bacillus subtilis* (k1) on various nutrient cultures supplemented with potatoes and sugar beets as carbon sources (Each graph is presented with mean values and their standard error from 4-replicates with significances at 0.05 level (5%) and with a,b,c,d ... letters for DMR test).

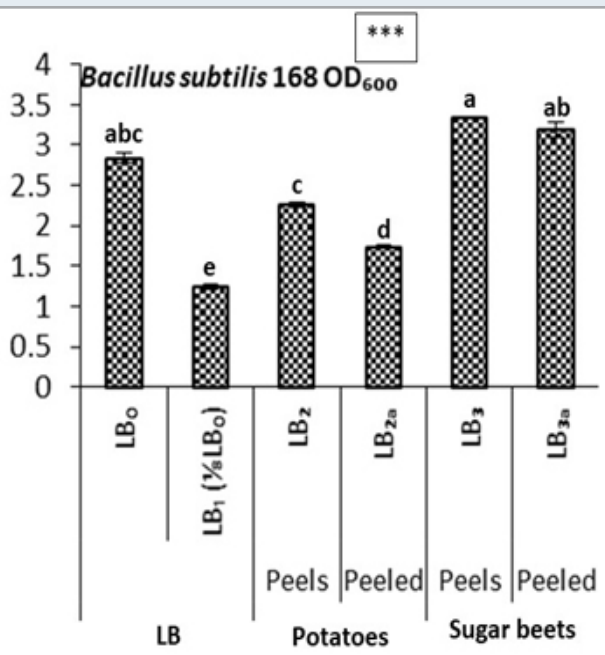
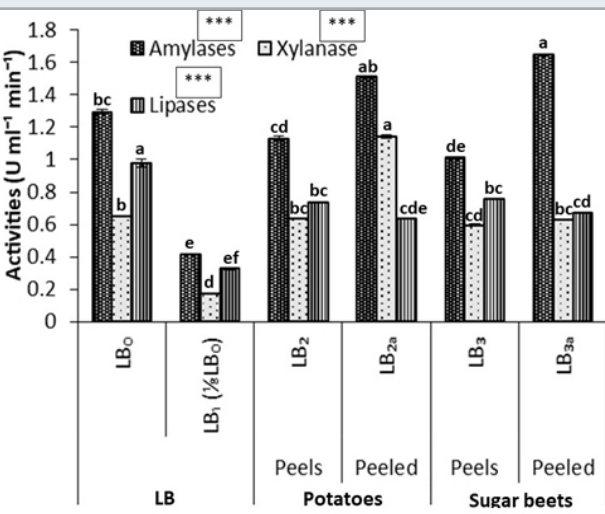


Figure 3: Comparative crude enzyme activities in the supernatant of *Bacillus subtilis* (k1) culture supplemented with potatoes and sugar beets as carbon sources. Each graph is presented with mean values and their standard error from 4-replicates with significances at 0.05 level (5%) and with a,b,c,d ... letters for DMR test.



Biochemical analysis of fermented supernatant: A number of biochemical testes performed on the fermented supernatant of *Bacillus subtilis*. Like as the total sugars analysed by mixing 1 ml supernatant with 2.50 ml concentrated H₂SO₄ and 5 µl 80 % phenol in a clean and dried glass test tube. This mixture was allowed to stand at room temperature for minimum 10 min than its absorbance was read at OD485 (Dubois et al., 1956). Similarly, reducing sugars were also measured by mixing 1 ml sample with 2.0 ml DNS (3, 5-Dinitrosalicylic acid) reagent than mixture was heated for 15 min in boiling water-bath. After that its OD540 was read (Aakash and Richa, 2020). Furthermore, total proteins were also determined with following Lovrien and Matulis (2004) method. Exact 2.5 ml alkaline copper reagents mixed with 1 ml supernatant. The mixture was mixed thoroughly and incubated at room temperature for 10 min than 0.25 ml folin reagents (1:1, w/v) poured into the wall of the test tube and than its OD750 was read. Meanwhile the free prolines (Schweet, 1954), glycinebetaine (Grieve and Grattan, 1983), total flavonoids (Woisky and Salatino, 1998), total phenolics (John et al., 2014), ascorbic acid (Tabata and Morita, 1997) and antioxidants (Prieto et al., 1999) were also analyzed in the same *Bacillus* culture supernatants by following the respective reported methods.

The phosphate contents analyzed by mixing the 0.5 ml ammonium molybdate with 3 ml concentrated H₂SO₄ then 4 ml test sample added. After that 1 ml 0.05 M sodium sulphate also added. Its OD was read at 715 nm (Mahadevaiah et al., 2007). The methionines were also analyzed by mixing 0.2 ml 5 N NaOH with 1 ml test samples than 0.02 ml 10% Nitro-Prusside was added. After 10 min, 0.4 ml 3% aqueous glycine poured to the reaction mixture. It was incubated for 10 min at room temperature than finally 0.4 ml ortho-phosphoric acid added. Absorbance was read against blank at 540 nm after 5 min of incubation at room temperature (Gensch and Higuchi, 1967; Lavine, 1943). The citric acid quantified with titration method (Aguiar et al., 2005) and its quantity was calculated by applying this formula; Aceti acid (g 100 ml⁻¹) = Volume of NaOH used*(0.03)*20.

The total fructose contents were quantified in the supernatant by mixing 3.5 ml 30 % HCl. The mixture was incubated in ice-bath for 10 min than 1 ml sample added. After 5 min of incubation in ice-bath 0.5 ml resorcinol thiourea was added. After that the reaction mixture was incubated at 80°C for 10 min than it was cool down under running tap water and its absorbance was read at 520 nm (Arsenault and Yaphe, 1966).

Measurements of enzyme activity: The supernatant of agro-fermented cultures of *Bacillus subtilis* was used as a crude enzyme mixture for the analysis of different enzymes activities. For the analysis of the *amylases* activities, its 1 ml used crude enzyme mixture mixed with 1 ml 0.5% starch fresh solution. The reaction mixture was incubated for 15 min at 37°C and than 2 ml DNS added to develop product complex and to stop

Among the other metabolites, production of total sugars sharply high in LB₃, LB₂ and LB_{2,a}, while it is declined in LB₀. The reducing sugars and total proteins observed higher in LB₃ culture. The production of fructose noted higher in LB₂ and LB₃, citric acid in LB₂, ascorbic acid LB₂ and LB₃ (Table 2) cultures. The free prolines and glycinebetain were observed higher in LB₂ and LB_{2,a}, while phenolics in LB₃ and LB₀ cultures. Production of flavonoids increased in LB₂, antioxidants and methionine in LB₃ cultures.

The *B. subtilis* has showed the production of various extra-cellular enzymes, which can lead to degrade the agro-based substrates (Meng et al., 2014). Similarly, amylases, xylanases, lipases and pectinases are also produced among other extra-cellular productions in the cultures when rotten potatoes and sugar beets are used as carbon source. Both are rich in starch, which may be the basic source for the production of fructose/glucose syrup. Meanwhile, carbohydrates based agricultural products like starch from potato as well as sugar beets occur abundantly. The amylases have shown very drastic activities among the peeled-potatoes and sugar beets, while xylanases and pectinases are very well performing in the cultures supplemented with peels of both agro-wastes as the carbon source. This differential formate of activities of various enzymes is due to the presence of their respective substrate in the plant organ (Haq et al., 2017 and 2018).

From the results as shown in figure 3 about the pectinases production on various cultures and its stability at under different temperature conditions for 30 min. Maximum activities of pectinases observed at 50°C in LB₀, which is declined in LB₂ (nutrient deficit medium) then slightly increased in LB₁ and LB₃, while it was relatively lower in LB₂ and in LB₃ cultures. Almost similar but directly proportional pattern of activities observed among the cultures at 37°C and 25°C (Fig 3). Overall, pectinases showed best activities at 50°C in all cultures and it could be suggested that the production of pectinases are higher where its substrate is present in the medium (Amin et al., 2013). Similarly, the pectinases of *A. fumigates* and *P. italicum* remain stable even upto 60°C while remained highest active at 50°C (Phutela et al., 2005).

CONCLUSION

The rotten vegetables especially potatoes and sugar beets are rich with starch and other carbohydrates i.e. cellulose and lignin. These renewable agriculture wastes are available as the cheapest carbon source for the growth of fermentation microorganisms for the production of industrial hydrolytic enzymes. The *Bacillus subtilis* (k1) is one safe fermentation organism can grow on these substrates with abundant production of industrial enzymes especially amylases, pectinases and xylanases. The same setup could be best for the production of glucose syrup as the potatoes and sugar beets are rich in starch, which is major key source of sucrose.

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