INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 12, No. 3, p. 285-293, 2018

RESEARCH PAPER OPEN ACCESS

Extra-cellular productions of *amylases* and *proteases* in rotten potato based cultures of *Bacillus subtilis*

Ikram-ul Haq*, Asra Maher, Nazia Parveen Gill[1], Ghulam Asghar Maka[2], Urooj Fatima[3], Kazim Ali, Ghulam Qadir

Institute of Biotechnology and Genetic Engineering (IBGE), University of Sindh, Jamshoro, Pakistan.

[1]Department of Statistics, University of Sindh, Jamshoro, Pakistan

[2]Institute of Microbiology, University of Sindh, Jamshoro, Pakistan.

[3]Department of Biomedical Engineering, Mehran University of Engineering and Technology, Jamshoro, Pakistan

Key words: *Bacillus subtilis*, Rotten potatoes, *α-amylase*, *Proteases*, Fermentation, Food industry wastes

http://dx.doi.org/10.12692/ijb/12.3.285-293 Article published on March 30, 2018

Abstract

The gram-positive bacteria like as *Bacillus* sp., are non-pathogenic and useful workhorse in industrial fermentations. In present study, extracts of rotten potato peels and peeled off potato starch used as fermentation substrate in combination with TY-medium (v/v) for *B. subtilis* 168 culture. Cultures harvested after 42 hrs and 62 hrs of incubation at 37°C with 250 rpm shaking incubator. Maximum cells observed in control LB_0 (TY-medium) and LB_2 (¼ TY+¼ potato peels or skin) cultures at 42 hrs, while in 2nd harvest it increased significantly in LB_2 and LB_3 (¼ TY+¼ peeled off potato starch), while minimum in LB_4 (¾ dH_2O+¼peels or skin) and LB_5 (¾ dH_2O+¼peeled off potato starch mixed). Highest reducing sugars measured in supernatant of LB_4 and then in LB_2 cultures (62 hrs), while minimum in LB_1 culture (42 hrs) significantly. Extracellular proteins noted higher in 2nd harvest of LB_2 and LB_4 (at 62 hrs) significantly, while minimum in LB_1 (at 42 hrs, $p \leq 0.05$). The *α-amylase* activities observed higher in all cultures at 1st harvest (at 42 hrs) except LB_1 (¼ TY-medium), while it decreased in LB_0, LB_1, LB_3 and LB_5 and increased in LB_2 and LB_4 cultures (62 hrs) timely. The *protease* activities remained almost same at 42 hrs ($p \leq 0.05$), while increased in LB_3 and LB_4 cultures (62 hrs). Both substrates have performed differentially, while potato peels has performed best. It is being cheapest agro-industrial waste and useful substrate could be recommended for production of extracellular heterologous proteins for industrial usages.

* **Corresponding Author:** Ikram-ul Haq ✉ rao.ikram@yahoo.com

Introduction

The agricultural raw material to food processing industries produces large quantity of wastes rich in renewable carbohydrates and organic nitrogenous compounds. Almost 1.5 million tons starch is produced from these wastes annually and its more than 60% is gained from potato plant and potato tubers (Goyal *et al.*, 2005; Grommers and van der Krogt, 2009; Oda *et al.*, 2002). Fermentation is an emerging and fast growing field of research for the production of large number heterologous proteins from renewable carbohydrates containing plant waste materials (Alibardi and Cossu, 2016; Yang *et al.*, 2016). Overall utilization of microbial fermentation on renewable agricultural wastes is a revolutionary technology for economic bio-production of valuable organic compounds and proteins (Becker *et al.*, 2015; Sauer *et al.*, 2008).

During processing of potatoes either domestic or industrial, they are peel off its digestible (by non-ruminants) too fibrous layer (Simpson *et al.*, 2012). The potato peel waste (PPW) is around 15-40% (Arapoglou *et al.*, 2009; Grassin and Coutel, 2009), which is rich with lipids, proteins, lignin and polysaccharides and valuable cheapest fermentation substrate (Khawla *et al.*, 2014; Wu *et al.*, 2012).

Since enzymes are capable to catalyze all chemical reactions, the industrial catalysis is increasingly dependent on enzymes, which can perform specific conversions of compounds within seconds to minutes without other interference (Dalby, 2003; Otten and Quax, 2005). Further organic solvents based enzymatic reactions enhances non-polar solubility of substrates, inhibits hydrolysis of substrate and prevent microbial contamination (Badoei-Dalfard and Karami, 2013; Doukyu and Ogino, 2010; Li *et al.*, 2009). To fulfill the industrial requirement, a major difficulty is downstream purification of the catalysts from cell culture after fermentation processes.

For its carbon and nitrogen sources, utilization of agro-industrial residues are promising low cost reservoirs (Schievano *et al.*, 2009). Like as most enzymes isolated from soil and water bacteria display desired activity, while remains unsuitable for industrial use (Griffiths and Tawfik, 2000; Marrs *et al.*, 1999; Otten and Quax, 2005). Such suitable enzymes with industrial desired activity may be obtained with optimization of process conditions or molecular protein engineering (Arnold, 1998; Dalby, 2003; Otten and Quax, 2005) as well as from microbial cells growing under differential environmental conditions (Dalby, 2003; Schoemaker *et al.*, 2003).

Fermentation of renewable agro-industrial wastes is a hydrolysis processes with most dominant hydrolases like as *amylases* (starch hydrolase) and *proteases* (protein hydrolases). The use of peels and peeled off rotten potatoes as fermentation substrate, which offers an attractive alternative source of carbon and nitrogen for low cost secretion or production of *amylases* and *proteases* with *Bacillus subtilis* 168. There no information is available regarding to the production of amylases and proteases from rotten potato wastes by *Bacillus* strains. All three forms of *amylases* break the starch molecules, while α-amylase is most important food industrial enzyme (Gupta *et al.*, 2003). Among the proteases, neutral proteases are food industrial enzyme especially for meat tenderizer, formation of protein hydrolysates or sweetener phenylalanine etc (Contesini *et al.*, 2017; Hmidet *et al.*, 2010; Jamrath *et al.*, 2012).

Production costs of hydrolases depends on commercial medium type, which generally comprised on complex carbon and or nitrogen containing molecules substantially contribute towards the enzyme's cost (Renge *et al.*, 2012). It is almost 70% of the cost of extracellular enzyme fermentations, while utilization of agricultural wastes based medium reduce upto 50% costs of medium for enzyme production (Anwar *et al.*, 2014; Jamrath *et al.*, 2012; Strauss *et al.*, 2001). By keeping under consideration above reports, goal of present work is conducted for the evaluation of agricultural wastes like as rotten potatoes. The peels and peeled off rotten potatoes also rich in starch and many organic nitrogen containing compounds. It could be used as a substitute of carbon and nitrogen source of bacterial fermentation medium.

Materials and methods

Micro-organism and cell cultures

The frozen glycerol stock cell-culture (16%) of *Bacillus subtilis* 168 is reactivated (Cook, 1996). Exact 2.0 µL glycerol stock inoculated in bacterial growth TY medium (Sambrook, Fritsch, & Maniatis, 1989). Cell culture incubated at 37°C with 250 rpm orbital shaking growth incubator for overnight. The pre-culture re-inoculated at the rate to develop an approximate initial OD 0.01 (600nm) in fresh TY medium and its various mixtures rotten potato extracts (Table 1). In total six medium treatments maintained and each treatment has three replicates. The TY medium mixes contain the waste rotten potato materials in place of carbon-nitrogen sources of TY medium. The pH of each medium adjusted to 7.0 before fermentation medium inoculation with *Bacillus* cells. The volume of each nutrient medium maintained to 25ml and after incubation cultures harvested after a time interval of 42 and 62 hrs.

Preparation of rotten potato's extracts

Almost partially rotten potatoes collected from local market and washed with tap-water and rinsed in 70% ethanol for 2 minutes. These cleaned potato-tubers peeled-off and both peels and peeled off potatoes material chapped into smaller pieces and weighed separately. Each grinded with electric grinder and past of peels mixed in triple volume and peeled off potatoes in double volume distilled water (dH_2O). Autoclaved slurry (121°C, 15lbs/cm², 20min) filtered with sterile muslin-cloth. This filtrate mixed with TY-medium (Table 1) and different bacterial fermentation medium maintained in triplicate.

Table 1. Composition of various *Bacillus subtilis* growth cultures supplemented with rotten potato as a carbon source.

SN	Medium	Composition of medium
01.	LB_0	TY medium: 10 g L^{-1} trypton, 5 g L^{-1} NaCl and 5 g L^{-1} yeast extract
02.	LB_1	¼ volume TY medium + ¾ volume dH_2O
03.	LB_2	¼ volume TY medium + ¼ volume peel extract + ½ volume dH_2O
04.	LB_3	¼ volume TY medium + ¼ volume peeled potato extract + ½ volume dH_2O
05.	LB_4	¼ volume peel extract + ¾ volume dH_2O
06.	LB_5	¼ volume peeled potato extract + ¾ volume dH_2O

Note: Volume of each culture was 25 ml and maintained in 200 ml conical volumetric flask

Biochemical analysis and enzyme activities

Cultures on each medium were maintained after bacterial inoculation up-to 62 hrs under controlled conditions. These cultures were harvested at two time intervals i.e. first harvest at 42 hrs and second at 62 hrs after incubation. At each harvest, OD600 of cultures was taken first than they were centrifuged (5,000 rpm, 10min). Its cell pellet was frozen at -20°C and supernatant stored at 4°C. The supernatant was subjected for the estimation of different organic substances produced by *Bacillus subtilis* 168.

Estimation of total proteins and reducing sugars

The culture supernatant is used for the measurement of total proteins with a method designed by Lowry's group (1951). The 0.5ml supernatant and 2.5ml alkaline copper reagent were mixed thoroughly and allowed to stand at room temperature for 10 minutes. Exact 0.25ml follin ciocalteau reagent poured than after 30min OD was read at 750nm. For the determination of reducing sugars, one milliliter supernatant mixed with 1ml 2, 6-dinitro salicylic acid (DNS). Mixture was heated in boiling H_2O for 5min than reaction was cool down and absorbance was read at 540nm (Miller, 1959).

Analysis of α-amylase activity

The 0.3ml of supernatant contains crude α-*amylase* was mixed with 0.5ml 1% soluble starch (w/v) as substrate (0.05M phosphate buffer, pH 6.5). The mixture was incubated at 45°C for 10min to stop the reaction and cold down at room temperature. The total released reducing sugars were quantified with colorimetric method by adding 1ml 3,5-dinitrosalicylic acid reagent against D-glucose standard curve (Miller, 1959). Enzyme activity per unit (U) defined as amount of enzyme involved to release 1.0µmol glucose per minute of time interval.

Protease activity measurements

The *protease* activity was measured by following caseinolytic activity method (Genckal and Tari, 2006). Briefly, 0.5ml supernatant and 2ml casein solution [10mm sodium phosphate, 150mm NaCl, pH 7.4, 1% casein (w/v)] mixed. The reaction mixture was incubated at 37˚C for 10min than reaction terminated with 2.5ml 0.1M trichloroacetic acid (TCA).

After 30min, mixture was centrifuged (10,000 rpm, 10min, 4˚C). Its 2.5ml mixed in 3ml Na_2CO_3 (0.5M) and 0.5ml Folin Ciocalteu (1N) reagent. After 10min, absorbance was read at 660nm against the tyrosine. Enzyme activity was defined amount of enzyme useful for the formation of 1µmol tyrosine per min is considered as one unit (U).

Statistical analysis

The cultures of each medium arranged for six medium treatments and each with three replicates. Collected data for each treatment of this experiment are subjected for comparative data significance analysis at the level of 5% differences. It is computed with *COSTAT* (*CoHort* software, Berkeley, USA) computer based statistical package.

Results and discussion

The *Bacillus subtilis* has been pronounced as non-pathogenic soil bacteria found both live to dead bodies of animals and plants including air and water also (Margot and Karamata, 1988; Rosenberg *et al.,* 2012; Tam *et al*., 2006). The *Bacillus* species have ability to grow from normal to harsh environmental conditions due to its ability to secrete a large number of heterologous proteins.

The variant performances of different strains on different agro-industrial wastes are making their usefulness for industrial applications for the biosynthesis of large number of proteins in the form of antibiotics and enzymes including other vitamins and amino acids (Alina *et al*., 2015; Eppinger *et al*., 2011; Schallmey *et al*., 2004).

Large number of microbial flora like as bacteria, fungus and yeast had been used for the production of safe prebiotics to support human health (Aachary and Prapulla, 2011; Cutting, 2011; Duc *et al*., 2004). The *B. subtilis* grows in normal human gut and produces a number of proteins which involves in maintaining the population of gut-flora (Hong *et al*., 2009; Lee *et al*., 2010; Schäfer *et al*., 1996). Also it is performing the useful bio-tasks for the preparation of many fermented foods (Ikeda *et al*., 2006; Wang and Fung, 1996; Yang *et al*., 2016).

In present study, *B. subtilis* 168 has shown variant growth pattern in the cultures supplement with different nature of substrate (Table 1). The rotten potato, an agriculture waste is used for bacterial fermentation as carbon source. Both extracts collected after grinding of rotten potato's peels and peeled-off starch stuff separately.

The cultured cells are harvested after incubation of 42 hrs and 62 hrs at 37°C with 250 rpm shaking incubator. Maximum cell growth rate observed in control LB_0 (TY-medium) and potato peels (LB_2) cultures at 42 hrs. It is further exceeded significantly in LB_2 and LB_3, which observed at second harvest taken after 62 hrs of incubation (Fig 1a).

While LB_4 and LB_5 showed minimum cell multiplication, when both peels and peeled off starch mixed with distilled dH_2O. Overall peels or skin of potato has shown stability in cell growth either mixed in Ty-medium or dH_2O. Among the biochemical component analysis of culture supernatant, highest reducing sugars measured in LB_4 and then in LB_2 cultures harvest at 62 hrs of incubation ($p \leq 0.05$). Minimum reducing sugars also noted after 42 hrs of incubation in LB_1 cultures (Fig 1b).

All cultures a shown in table 1 presented higher extracellular protein contents in 2nd harvest, which has been taken after 62 hrs of incubation than 1st harvest at 42 hrs (Fig 1c). Comparatively, protein contents observed maximum in LB_2 and LB_4 (at 62 hrs) significantly, while minimum in LB_1 (at 42 hrs, $p \leq 0.05$).

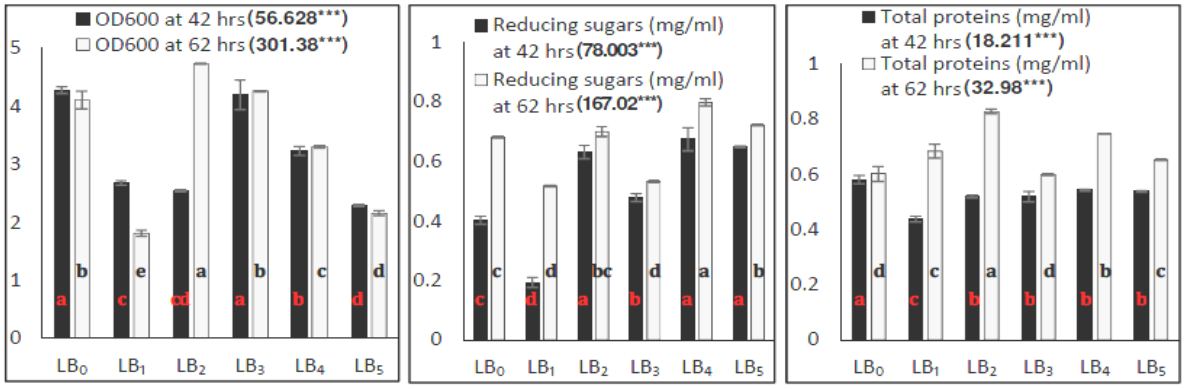

Fig. 1. Various attributes of cell growth cultures supplemented with rotten potato as fermentation substrate for *Bacillus subtilis* 168 (after 42 hrs and 62 hrs of incubation). a. Cell multiplication rates at OD600; b. Total reducing sugars (mg/ml); c. Total extracellular protein contents (mg/ml).

The *B. subtilis* 168 has been considered as most important fermentation bacteria and being a source species of *α-amylase* and *proteases*. It is useful for enzyme production frequently with sub-merged fermentation method (Windish and Mhatre, 1965).

Significant and differential activities of α-*amylase* measured from LB_0 to LB_5 cultures (Fig 2a). Maximum *α-amylase* activities observed in all cultures (except LB_1) at 1st harvest (at 42 hrs), while with the passage of time it decreased in LB_0, LB_1, LB_3 and LB_5 but it increased significantly among the LB_2 and LB_4 cultures harvested at 62 hrs of incubation.

Similarly *protease* activities also measured in the supernatant collected at both harvests (42hrs and 62 hrs). Almost same but variant *proteases* activities noted at 42 hrs of culture incubation ($p \leq 0.05$). The significant increase in proteases activity observed in all cultures, while it noted maximum among LB_3 and LB_4 culture at 62 hrs of incubation (Fig 2b). It could be an indication especially at growth stationary or cell decline phase. Both of higher enzymatic activities among the cultures and increasing trend of reducing sugars are involved to maintain the specific cellular adjustment for the survival of cells under nutrient deficit conditions.

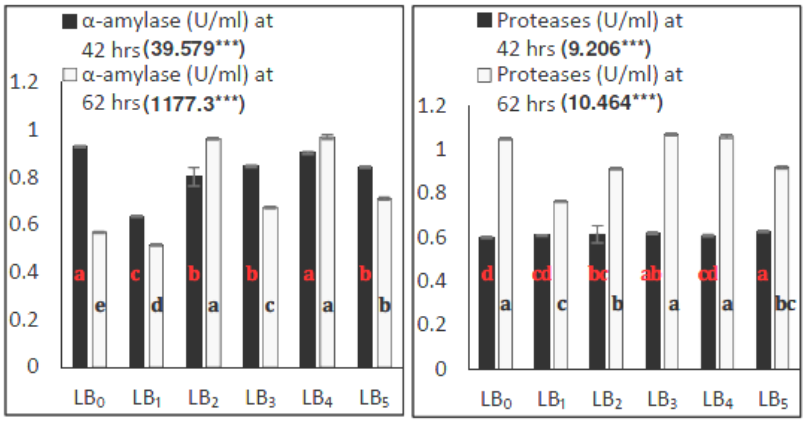

Fig. 2. The α-*amylases* activities in *Bacillus subtilis* 168 cultures supplemented with rotten potato as a fermentation substrate after 42 hrs and 62 hrs. a. α-*amylases* activities (U/ml); b. alkaline *proteases* activities (U/ml).

The *amylases* are mainly employed for liquefaction of starch for the production of mixtures of simpler oligosaccharides in the form of maltose, fructose and their syrups also (Benjamin *et al.*, 2013; Taniguchi and Honnda, 2009).

These are finally degraded oligo- and monosaccharaides, which are easily absorbable by fermentation organism for its cell growth and further secretion of same amylases or many by-products (Pandey *et al.*, 2000).

Similarly proteases are very important industrial (pharmacy, leather, food and waste processors) enzymes but out of plants, animals and fungal proteases the bacterial especially of *Bacillus* sp., are extra-cellular specific *proteases* producers (Durham *et al*., 1987; Takimura *et al*., 2007). Both *amylases* and *protease* are produced by *Bacillus subtilis* 168 in liquid medium supplemented with different forms rotten potatoes as a substrate (Fig 2). The rotten potatoes are available freely from the agriculture-fields as well as its processing industries. It may be recommended as fermentation substrate for microbial fermentation for extracellular heterologous industrial enzymes or proteins productions.

Conclusions

The agricultural waste especially peels or skins of fruits and vegetables are discarded during their processing either to use at house or industries level. It is an ongoing process, which is due to difficulty in its digestion by human stomach.

This outer layer of plant fruits has a very complex structure and it is comprised on complex molecules carbohydrates, lipids and proteins. The *Bacillus subtilis* has ability to liquefy it into simple carbon molecules with its extracellular enzymes. Similar phenomena have observed in present experiment, maximum amylases and protease production observed in potato peels waste extract than potato starch. Even at later stages of culture cell's growth is measured maximum among the cultures supplemented with potato skin or skin wastes.

The peels or skin wastes of potato or even other vegetables or fruits could be a useful and economic carbon source of fermentation industry for the production various extra- or intracellular proteins.

Acknowledgements

Authors are thankful University of Sindh, Jamshoro, Pakistan for provision of funds for completion of this work. Also we would like to thanks to staff members of IBGE for providing potato stuff and special thanks to Mr. Aftab Hussain for his helpful engagement during biochemical contents analysis with undergraduate students.

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