



Molecular characteristic of α -AMYLASE enzymes producing from *Bacillus licheniformis* (JQ946317) using solid state fermentation



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ABSTRACT

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units. In the present studies bacterial strain were isolated from the soil samples and identified as *Bacillus licheniformis* (JQ946317). Maximum α -amylase activity was obtained at pH 7.0, 35 °C after 48 h of incubation. The enzyme was purified using acetone precipitation and DEAE- Sphadex A50 column chromatography and the molecular weight of 63 kDa was confirmed by SDS-PAGE. This enzyme exhibited maximum activity at pH 7.0, performed stability over a broad range of pH 6–8 and was optimally active at 35 °C. The enzyme activity was highest at 48 h of incubation as a carbon sources preparation had a strong digesting ability towards various agricultural waste and wheat bran showed highest enzyme. The purification and characterization of α -amylase from a newly isolated *Bacillus licheniformis* (JQ946317).

1. Introduction

The α -amylases (endo-1, 4- α -D-Glucan glucohydrolase; E.C. 3.2.1.1) catalyze the (a-1 \rightarrow 4) O-linked glycosidic bond present in soluble starch (i.e. amylose) and hydrolyze in a random fashion to produce reducing sugars in the α -configuration (Kharkrang and Ambasht, 2012). The α -amylase is calcium metalloenzymes, completely unable function in the absence of calcium. α -amylase tends to be fast acting than β -amylase because it can act anywhere on the substrate. In human physiology, both the salivary and pancreatic amylase is α -amylase (Das et al., 2011). The stability and activity of α -amylases that are generally used in starch hydrolysis are Ca^{2+} dependent. Since Ca^{2+} ions inhibit glucose isomerase, the added Ca^{2+} must be removed from the product stream using ion-exchangers before the isomerization step from glucose to fructose. Consequently, the demand for Ca^{2+} independent α -amylases has increased (Antrim, 2007). (see Tables 1–4, Figs. 1–4)

Enzymes are among the most important products obtained for human needs through microbial sources. The α -amylase is being isolated from a number of microbial sources and every enzyme moiety isolated from various microbial sources is unique in their characteristics (Reilly, 1991). Enzymes have attracted attention of researchers all over the world because of wide range of physiological, analytical and industrial application; especially from microorganisms because of their broad biochemical diversity, feasibility of mass culture and ease of

genetic manipulation (Chakrabortya et al., 2009). Enzymes used in food processing have historically been considered non-toxic. Some characteristics arising from their chemical nature and source, such as allergenicity, activity related toxicity, residual microbiological activity and chemical toxicity are, however, of concern. These attributes of concern must be addressed in light of the growing complexity and sophistication of the methodologies used in the production of food-grade enzymes. Safety evaluation of all food enzymes including those produced by GM micro-organisms is essential if consumer safety is to be assured (Spok, 2006). Among these, proteases and amylases are of significant industrial importance. In particular, amylases comprise 30% of the world's industrial enzyme production (Hmidet et al., 2010). Some *Bacillus* strain produce the enzymes in the exponential phase, whereas some other in the stationary phase, though the pattern of growth and enzymes profile of *Bacillus* species have similarities, the optimize condition for the enzymes differs widely depending upon the strain (Mulimani et al., 2006).

Mutagenesis and genetic recombination techniques using protoplast fusion transformation have been used widely by several workers as a tool of protein engineering to achieve strain with higher enzymes productivity or desired characters. (Mitchell et al., 1985).

Solid state fermentation (SSF) has tremendous potential for the production of enzymes not only for laboratory but also for commercial production (Goes et al., 1999). For the modern biotechnology alpha

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Table 1
Effect of incubation period for amylase production.

Days	Wild			Mutant		
	Black Bran (U/ml)	Rice Bran (U/ml)	Wheat Bran (U/ml)	Black Bran (U/ml)	Rice Bran (U/ml)	Wheat Bran (U/ml)
1	14.26	28.32	35.75	23.77	47.2	59.58
2	21.17	31.3	44.12	35.29	52.17	73.53
3	23.28	32.3	38.58	38.8	53.84	64.3
4	21.32	29.7	37.36	35.53	49.5	62.27
5	18.46	30.47	34.11	37	50.79	56.85
6	18.66	31.12	34.34	31.16	51.86	57.24

Table 2
Effect of pH on Amylase Activity.

PH	Wild			Mutant		
	Black Bran(U/ML)	Rice Bran (U/ML)	Wheat Bran (U/ML)	Black Bran(U/ML)	Rice Bran (U/ML)	Wheat Bran (U/ML)
6	15.35	26.9	34.13	22.58	44.84	56.88
6.5	22.16	29.74	34.4	52.53	49.56	57.34
7	31.51	30.69	42.91	36.86	51.15	69.85
7.5	20.25	36.65	36.65	33.75	47.03	61.08
8	21.09	28.95	35.5	35.15	48.25	59.17

Table 3
Effect of temperature on amylase activity.

Temperature	Wild			Mutant		
	Black Bran U/ML	Rice Bran U/ML	Wheat bran U/ML	Black Bran U/ML	Rice Bran U/ML	Wheat Bran U/ML
30 °C	13.84	28.73	34.62	23.06	45.78	57.7
35 °C	20.52	30.36	42.79	34.2	50.6	71.32
40 °C	22.58	31.3	37.42	37.64	52.54	62.37
45 °C	20.68	28.8	36.46	34.64	48	60.4

amylase are now gaining importance in biopharmaceutical applications. Still these application in the food and starch based industries is the major market and thus the demand of alpha amylase would always be high in these sector (Rani et al., 2003).

In the present study scenario, applied with industrial processes such as in food, detergents, and textiles and in paper industry, for the hydrolysis of starch. They can also be of potential use in the pharmaceutical and fine chemical industries (Kandra, 2003). Based on these applications, the present study shows that the industrial important microorganism such as *Bacillus sp.* and their improvement of α -amylase production for various substrates using solid state fermentation process.

2. Materials and methods

2.1. Primary screening of α -amylase producing bacteria

The collected soil samples were diluted by serial dilution technique.

Table 4
Purification table.

step	Total protein (ug/ml)	Total activity (U/ml)	Specific activity (unit/ml/ug protein)	yield	Protein fold
crude	11.5	8550	735.16	100	1
Acetone precipitation	10.5	8500	798.87	94.41	1.08
dialysis	6,3	6000	952.38	70.17	1.29
Pure	3.5	4000	1250	46.78	1.70

Effect of Incubation Period

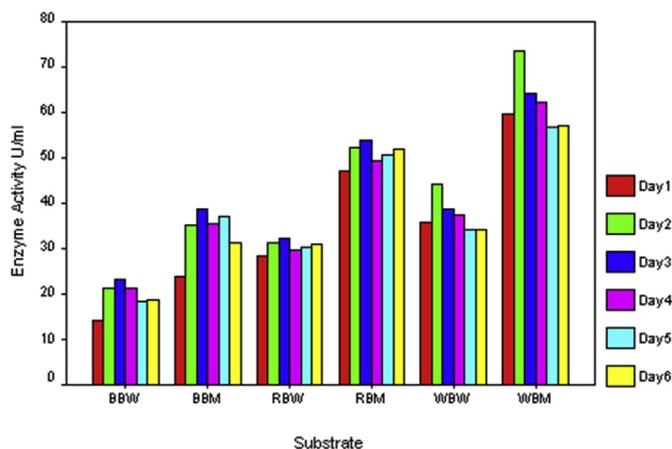


Fig. 1. Enzyme production on different incubation period
The figure above shows the bacterial alpha-amylase activity at different pH gradients using DNS alpha-amylase activity assay method. The enzyme shows maximum activity at pH 7.

Effect of Different pH

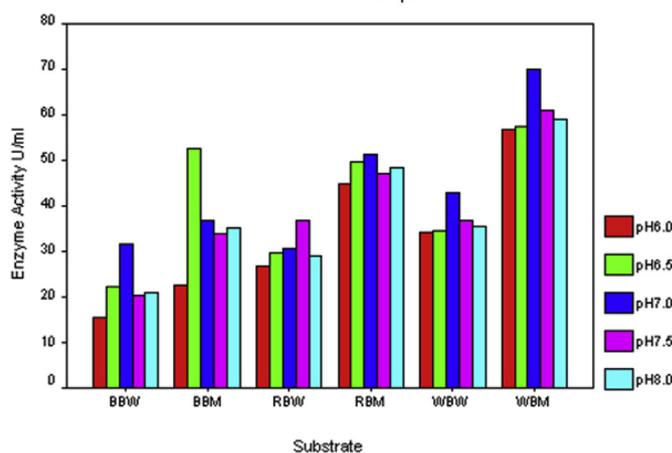


Fig. 2. Enzyme production on different pH.

The diluted samples of 10^{-4} to 10^{-6} (0.1 ml) were spread with L-shaped glass rod by spread plate technique on the starch agar plates. The isolated strains were maintained on nutrient agar slants and stored in refrigerator at 4 °C. It was performed using agar plates amended with starch. The agar plates were prepared amended 2% of starch with 1.5% of agar. After agar solidification, culture was streaked in the starch agar plates with starch as the only carbon source and incubated at 37 °C for 24–48 h. The agar plate was flooded 1% of grams iodine solution observation was made to see the hydrolytic zone around the streaked area on the plate. The colonies showed the greater zone of clearance in starch agar plate were maintained on to starch agar plate as well as to nutrient agar slants(Amoozegar et al., 2003).

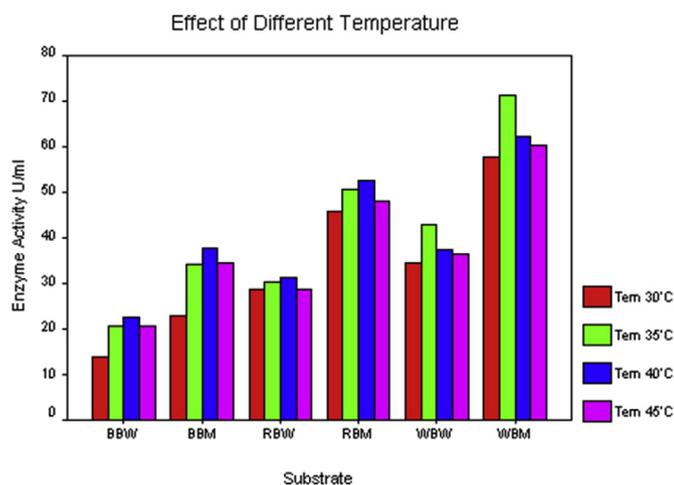


Fig. 3. Enzyme production on different temperature.

M- Ladder S- Sample



Fig. 4. Protein profile in SDS- PAGE.

2.2. Estimation of amylase by DNS method

Determination of reducing sugars was carried out by dinitrosalicylic acid (DNS) method. To prepare the reaction mixture 1 mL of supernatant was mixed with 1 ml of solubilized starch solution and then incubated at 60 °C for 10 min. The reaction was stopped by adding 2 ml of dinitrosalicylic acid (DNS) reagent. The mixture was cooled in an ice water bath for 10–15 min and then centrifuged 5000 × g for 5 min at 4 °C. The absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of amylase needed to produce 1 μmol of maltose per min under the assay conditions. (Miller, 1959).

2.3. Ultraviolet treatment to isolate mutants

Ultraviolet treatment to isolate mutants 24 h old bacterial culture prepared in nutrient broth was aseptically centrifuged at 5000 rpm for 15 min. The bacterial sediment was made suspension with sterile saline water. Ten ml of diluted suspension was transferred to sterilized petriplates placed under the UV lamp (emitting the energy of 1.6×10^2 J/m²/s) for 80 min (42% survival times) to 120 min. The bacterial suspension (0.5 ml) was collected after every 10 min interval and transferred to solidified starch agar petriplates (180 × 20 mm BOROSIL) by spread plate technique. The plates were invertedly incubated in an incubator at 37 °C for 24 h (Ashraf et al., 2001).

Physical mutagen treatment the mutant colonies showing more than zone of starch hydrolysis in starch agar medium were isolated in pure culture (streak plate technique) on nutrient agar plates. The mutants were single line streaked over starch agar plates to confirm the size of

the zone of starch hydrolysis. The confirmed mutants were subjected to Solid state fermentation at 120 rpm in rotary shaking incubator in different fermentation medium (100 ml) in Erlenmeyer flasks. The α-amylase activity was assayed by DNS method.

2.4. Substrates

Different agro-industrial waste materials (Black Bran, Rice Bran and Wheat Bran) were collected from the local market and processed and stored till further use.

2.5. Solid-state fermentation

Weigh 10 g of substrate were taken in 250 ml Erlenmeyer flasks and to this a predetermined quantity of water was added, mixed thoroughly and autoclaved at 121 °C for 15 min at 1 kgf/cm². After cooling the flasks to room temperature, the flasks were inoculated with one ml of 24 h grown culture broth under sterile conditions. The contents of the flasks were well mixed and incubated at ± 33 °C for predetermined time period.

2.6. Enzyme production

To study the efficacy of various carbon sources on α-amylase production, Black Bran, Rice Bran and Wheat Bran were selected as carbon sources. Starch (1% w/v) was replaced in the defined production medium with equal amounts of the various carbon sources to be tested. A volume of 150 ml of production media containing test carbon sources was inoculated at 1% and placed in 500-mL Erlenmeyer flasks for culture.

2.8. Effect of incubation period on amylase activity

100 ml of sterile production medium was prepared in different conical flask. Each flask was amended with different substrate such as wheat bran, rice bran and black bran. The flask were inoculated with 1 ml of inoculums broth and incubated at 37 °C for different incubation period (1, 2,3,4,5, and 6 days) every incubation period collected and then enzyme assay was determined.

2.9. Effect of PH on amylase activity

100 ml of sterile production medium was prepared in different conical flask. Each flask was amended with different pH such as 6, 6.5, 7, 7.5 and 8 using 0.1 N NaOH. After sterilization conical flask were inoculated with 1 ml of inoculums broth and incubated at 35 °C for 48 h. Then culture filtrate was collected and enzyme activity was determined.

2.10. Effect of temperature on amylase activity

100 ml of sterile production medium was prepared in different conical flask. Each flask was amended with different temperature using water bath & hot air oven. After sterilization conical flask were inoculated with 1 ml of inoculums broth and incubated for 48 h. Then culture filtrate was collected and enzyme activity was determined.

2.11. Protein estimation

The proteins were estimated by using Bradford (1976) method. The standard bovine serum (BSA) solution (stock solution = 1 mg/ml) of varying series of concentration (10 μg–100 μg) was taken and made up to 1 ml with distilled water in test tubes. The unknown samples, 1–5 μl, were taken and then made up to 1 ml with distilled water. To all the tubes 2.5 ml of Bradford reagent was added. After 2 min, optical density (O.D) at 595 nm was read using a ultra violet –visible (UV–Vis)

spectrophotometer (PerkinElmer, USA) and standard graph was plotted where x axis represented the concentration standard and y axis, the O. D of the standard.

2.12. Enzyme purification and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and molecular weight determination

α -Amylases produced from the parental and its mutant strain were purified by 95% Acetone precipitation and DEAE- Sphadex A 50 column chromatography. The molecular mass of the purified enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDSPAGE (Laemmli, 1970).

2.13. Bacterial DNA isolation and PCR amplification

Total bacterial genomic DNA was isolated from 1.5 ml of overnight bacterial culture. Collected the pellet and 10% SDS, lysozyme, Proteinase K and mixed well. incubate at 50 °C for 30 min. An equal amount of Phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well by gentle inversion and centrifuged. The supernatant was transferred to a fresh tube and DNA was precipitated by adding 3/4 volume of isopropanol. After centrifugation, the pellet was washed in 70% ethanol, dried and dissolved in 1X TE buffer. RNA was removed by RNase treatment. DNA was quantified by comparing with uncut λ DNA on the agarose gel, diluted to 12.5 ng $^{-1}$ and used in PCR.

The amplification of a DNA using 16S Forward primer (5'-AGAGT TTGATCMTGGCTCAG-3') and 16S Reverse primer (5'-AAGAGGTG-WTCCARCC-3'). Thermal cycling parameters were an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min.

2.14. Application of enzyme activity

2.14.1. Desizing of potato and maize

Weigh 10 gm of potato and maize in a beaker and add 1 ml of inoculums and made up to 10 ml with sodium acetate buffer and kept for 48 h at room temperature. After incubation the mixture was filtered by muslin cloth and the filtrate was air dried then the weight of the substance was measured.

2.14.1. Desizing of textile cloth

A stiffed piece of fabric cloth was weighed on electric balance. The cloth was then dipped in 100 ml of enzymatic solution (Ph-6) and placed in incubator at 37 °C for 1 h. Then the cloth was washed with distilled water and dried. After drying the cloth strip was weighed again. The percentage of removal of starch was calculated by the following formula. (Haq et al., 2011).

3. Result and discussion

3.1. Screening

The bacterial strains were isolated from the soil for the production of amylase. From the soil 6 bacterial colonies were isolated. After screening it was found that only 3 strains showed the positive result.

3.2. Strain improvement by mutagenesis with UV

The bacterial cells were exposed to UV irradiation for 25 min at a distance of 30 cm. The mutant was picked up from the nutrient broth agar plate having at least maximum number of survival. Better strain will selected based on the comparison with the parental strain on starch agar plate. The mutation and screening of industrially useful microorganisms are important for the successful development of the various strains required for the use in the fermentation industry. The

productivity of the parent strain *Bacillus* sp. was improved by the application of UV irradiation. The present studies showed that mutant strain produce high enzyme then the parent strain. Similarly, the work is done by (Demirkan et al., 2011) that mutant strain had more stability than the enzyme of the parental strain in alkaline conditions (85% at pH 8.0). Likewise B10 *Bacillus* sp produce more α - amylase in 48 h i. e 340U/ml, whereas the mutant M3 strain was produced more amylase at 30 °C at pH 7.0 in 48 h of incubation, where the mutant showed more α -amylase activity at the 32.5 °C at pH 7.0 in 48 h of incubation (Samrot and Vijay, 2009).

3.3. Effect of incubation period on amylase activity

Usually the growth of bacterium and the enzyme production were influenced by different carbon sources (Grata et al., 2010). In the present study also the amylase enzyme production was influenced by the different carbon sources. The carbon sources (monosaccharides or polysaccharides) are replaced with starch in the medium. The mutant strain showed highest α -amylase production on wheat bran as compared to wild strain at 73.53 U/ml at 48 h of incubation, similarly amylase activity increased steadily and reached maximum at 96 h in *P. fellutanum* (Kathiresan and Manivannan, 2006). Chimata et al. (2010) said that maximum amylase production was obtained from wheat bran as a substrate under solid state fermentation; the maximum production of α -amylase was at 20% wheat bran (Haq et al., 2012).

3.4. Effect of PH on amylase activity

The effect of pH buffer solution was also one of the important factors that must be studied to optimize the enzyme conditions. The difference in pH 6–8.0 was investigated. It was found that the optimum conditions of the pH buffer solution ranging from pH 6 to pH 8. However, it preferred to choose the buffer solution of pH 7. Highest enzyme activity was observed at pH 7. Similarly Vaseekaran et al., 2010 reported that *Bacillus lichenformis* produce high amylase activity at the neutral pH and *Stroptomyces aureofaciens* showed maximum amylase activity at pH 7.0 (Shatta et al., 1990), *Bacillus* sp. KR-8104 produces the maximum α -amylase and grows best at neutral pH. *A. flavus* produced more amylase under submerged aerobic fermentation by using *Cocos nucifera* meal as a substrate at pH 7 (Arunyasi et al., 2010).

3.5. Effect of temperature on amylase activity

Several strains in *Bacillus* genus have shown that the temperature for α -amylase activity ranges from 37 °C to 80 °C (Burhan et al., 2003). In the present study the optimal temperature for the amylase activity (71.32 U/ml) was determined at 48 h of incubation in the wheat bran as a carbon source similarly the maximum enzyme activity was produced (300 U/gds) under optimum an incubation temperature of 35 °C. (Sindhu et al., 2009), optimum temperature for *Bacillus* strain at 37 °C. (Mishra and Behara, 2008); 30 °C for *Aspergillus flavus*. (Sourav et al., 2011).

3.6. SDS –PAGE analysis

SDS page analysis is employed for confirming the macromolecule is α -amylase or different enzymes, supported the mass of the macromolecule is 68 KDa and that is high molecular masses of α -amylases from *lichenformis*, Similarly molecular mass was estimated to be around 60.5 Kda. In *P. camemberti* PL21 (Nouadri et al., 2010), although comparatively low molecular mass (38 KDa) of α -amylases (Jyoti et al., 2011) (see Table 3).

3.7. Purification table

The purification factor was (38.5-folds) and a recovery was (23.1%).

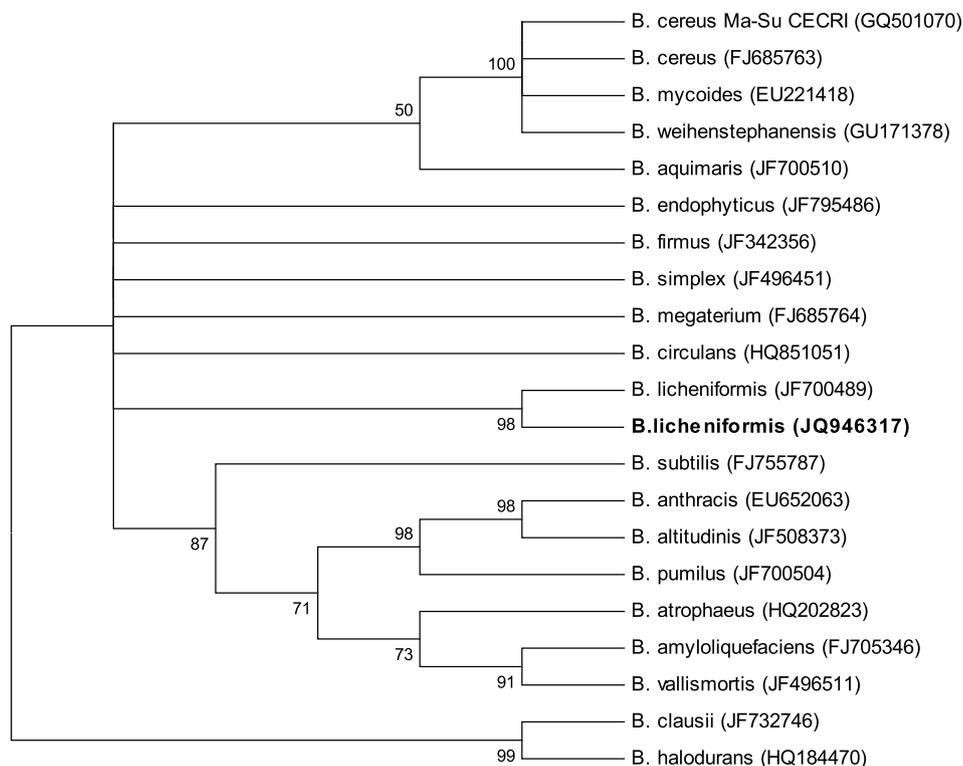


Fig. 5. Phylogenetic tree of 16S rRNA of the sample.

α -amylase maximum activity was (154.2 U/ml.mg protein) was reported by (Nouadri et al., 2010), in *P. camemberti* similarly present studies showed specific activity of purified enzyme is 1250 μ g/ml and the yield is 46.78. The protein fold of the purified amylase enzyme is 1.70 (Table 4).

3.8. Molecular characterization based on 16S rRNA sequence

PCR product was eluted and sequenced by Sanger method (see Fig. 4). After sequencing, the generated nucleotide sequences were edited and, the sequence was BLAST with NCBI after finding the similarity of sequence to confirm the genus and species. Based on this sequence we construct phylogenetic tree on using MEGA4.0 software (Tamura et al., 2011). The sequence was obtained was then blasted in NCBI data base. The length of the sequences is ~700 bp. Based on the 16S rRNA sequence and with the help of phylogenetic tree bacterium was confirmed as *Bacillus licheniformis* (Fig. 5), similarly (Arthi and Ramana, 2011) stated that confirmation of *B. cereus* with using NCBI BLAST (National centre for Biotechnology Information). The present study shows that maximum hydrolysis of potato starch (90%) and maize starch (60%) of 1 ml of crude enzymes.

4. Conclusion

Solid state fermentation (SSF) has tremendous potential for the production of enzymes not only for laboratory but also for commercial production. Based on these applications, the present study shows that the industrial important microorganism such as *Bacillus licheniformis* strain JQ946317. and their improvement of alpha amylase production for various substrates using solid state fermentation process. The *Bacillus licheniformis* strain JQ946317 extra-cellular α -amylase was purified by 95% Acetone precipitation and DEAE- Sphadex A 50 column chromatography. The purification factor was 1.70 and recovery was 46.78. α -amylase maximum activity was (1250.2 U/ml.mg protein). The molecular mass was estimated to be around 68 Kda. The enzyme was active with pH 7.0 and optimized temperature at 35°C at 48 h of

incubation. Hence, the enzyme isolated within the present study will become a decent candidate for industrial applications.

Conflicts of interest

The authors hereby declare that there is no conflict of interest.

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