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Purification and Biochemical Characterization of a Maltooligosaccharide Producing α-amylase from *Bacillus licheniformis* SKB 4

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Abstract: An extracellular amylase was purified upto homogeneity from the culture broth of *Bacillus licheniformis* SKB4 by a combination of 80% $(NH_4)_2SO_4$ precipitation, DEAE cellulose column chromatography, and sephadex G-100 gel filtration technique. The purified α-amylase exhibited specific activity of 827 U/mg with 64.8% yield having a molecular weight of 60 kDa and retained 80% of its original activity in the presence of 5 M NaCl solution. Starch is the best substrate (100%) for enzymatic digestion followed by amylopectin, potato starch, corn starch, amylose and glycogen. The non-inhibitory effect of β-specific inhibitor p-chloromercuro benzoate and iodoacetamide (10 mm), rapid blue loss percentage of starch-iodine complex and formation of α-anomeric products of starch hydrolysis indicated that the purified enzyme was an endo-attacking α-amylase. This enzyme hydrolyzes starch to maltooligosaccharides like materials

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maltotriose (G3), maltotetrose (G4), maltopentose (G5) at an early stage of the reaction which were further hydrolyzed to maltose. This salt-tolerant and maltooligomer producing α-amylase has commercial value, especially in the food industry.

Key words: Purification, amylase, maltooligosaccharide, *Bacillus licheniformis* SKB4

Introduction

Alpha amylase (EC 3.2.1.1, α-1,4-glucan-4-glucanohydrolase) randomly cleaves α-1,4 glycosidic bonds of starch, amylose, amylopectin and related polysaccharides in endo-attacking fashion and produces different sizes of oligosaccharides in an α-anomeric configuration [1]. Among various extracellular enzymes, α-amylase ranks first in terms of commercial exploitation.[2] Alpha amylases have a wide spectrum of applications including baking, brewing, detergent, textile, paper and distilling industry [3]. Amylases had been derived from many sources such as plants, animals and microbes, [4,5] but, microbial origin especially bacterial amylase generally meet industrial demands for their cost-effective production and thermostability [6,7]**.** Among the bacterial strains**,** *Bacillus licheniformis,* Gram positive rod shaped aerobic organism had a long history for production amylase in industrial sectors as well as research purpose.

The enzymes were purified from other protein and non-protein contaminants on the basis of their inherent properties like shape, size, charge, hydrophobicity, solubility and biological activity. Most of the time, a single chromatographic step was not sufficient to get the required level of purity and a multi-step purification system exhibited homogeneity of the protein. Generally, most of the amylases produced glucose and maltose as main products from starch hydrolysis, but only a few amylases can able to produce specific oligosaccharides like maltotriose, maltotetraose and maltopentaose [5,7,8,]. Maltose and maltooligosaccharides have applications in food, beverages and pharmaceutical industries. These oligosaccharides are highly water soluble and produce clear, tasty solutions, which are used as nutrients for infant and aged persons [9].

In our laboratory a thermostable, salt tolerant maltooligosaccharide producing organism had been isolated [8]. The present study deals with the purification and some specific characterization of α-amylase from SKB4.

Materials and Methods

Microorganism

Bacillus licheniformis SKB4, [8] a soil isolate was used for the present study.

Submerged fermentation for amylase production

Submerged fermentation was carried out for amylase production in 250 mL flask having 50 mL enriched medium (pH 6.5) which contains (w/v, g L^{-1}): starch, 5.0; peptone, 10.0; beef extract, 5.0; KH_2PO_4, 3.0; $MgSO_4$ 0.5; $CaCl_2$; 0.02. The medium was inoculated by addition of 1% (v/v) freshly prepared inoculum and incubated on a rotary shaker (120 rpm) at 42°C for 28 h. The culture supernatant was used to determine the amylase activity.

Purification of amylase

Culture broth was centrifuged (10,000 rpm, 4°C, 10 min) to remove cells and debris. The concentrated culture broth brought to 35.0% saturation with enzyme grade solid ammonium sulfate [$(NH_4)_2SO_4$] crystals followed by overnight incubation at 4°C. The supernatant was separated by centrifugation (10,000 rpm, 4°C, 10 min) and the precipitate was discarded after examining its amylolytic activity. Then 80% saturation was brought by adding solid $(NH_4)_2SO_4$ and allowed to keep at 4°C for overnight. The precipitate was collected by centrifugation (10,000 rpm for 30 min at 4°C) and dissolved in 10 mM Phosphate buffer (pH 6.5). To remove the ammonium sulphate, enzyme solution was dialyzed against the same buffer for 24 h at 4°C with a periodical change of buffer solution. The dialyzed enzyme was then applied to the DEAE cellulose column (Merck, Mumbai, India) pre-equilibrated with 10 mM Phosphate buffer (pH 6.5). The enzyme was eluted with a linear gradient of KCl (10-100 mM) at a flow rate of 1 mL/min. The active fractions in the flow through were collected (2.0 mL in each tube) and concentrated in lyophilizer, stored at 4°C for further purification. At the next stage, the amylase solution puts on Sephadex G-100 column (1.5×92 cm) that pre-equilibrated with 10 mM phosphate buffer (pH 6.5) and eluted with the same buffer at a flow rate of 0.5 mL/min. Fractions were collected and assayed for α-amylase activity. The enzyme fraction was concentrated through lyophilizer and stored at 4 (C for further use.

Estimation of protein

The protein content of enzyme solution in each step of purification was determined by measuring the absorbency at 280 nm (UV spectrophotometer, Hitachi, Japan, 2001), whenever necessary protein was also estimated following the method of Lowry et al. [10] using Bovine Serum Albumin (fraction V) as the standard.

Determination of molecular weight of amylase

The homogeneity of purified enzyme was tested through polyacrylamide gel electrophoresis (PAGE) in the presence and absence of sodium dodecyl sulphate (SDS) according to the method of Laemmli [11]. After electrophoresis, the gel was stained with Coomassie Brilliant Blue (R-250) dissolved in a water/methanol/acetic acid (50:40:10) solvent for 1 h, then distained in the same solvent. The zymogram was carried out according to Mitsunaga et al. [12]. After electrophoresis, the gel was incubated with 1% soluble starch solution in 10 mM phosphate buffer (pH 6.5) and then stained with iodine solution [1.3% iodine (I_2), w/v and 3.0% potassium iodide (KI), w/v] for detection of amylase activity and development of zymogram.

Assay of amylase

Amylase activity was determined by studying its saccharolytic properties according to Bernfield [13]. Briefly, the reaction mixture consisted 0.5 mL of 1% (w/v) starch, 0.4 mL of phosphate buffer (10 mM, pH 6.5) and 0.1 mL of enzyme solution and incubated for 5 min at 90°C. The reaction was stopped by the addition of 1 mL of 3, 5 dinitrosalicylate (DNS) reagent. The quantity of reducing sugar was measured colorimetrically at 530 nm using glucose as standard sugar. The

unit of amylase was defined as the amount of enzyme which produced 1 μmol of reducing sugar as glucose in 1 min under specified condition.

Determination of salt tolerance capacity

Enzyme was incubated in 10 mM phosphate buffer (pH 6.5) containing various concentration of NaCl (1 to 5 M) for 24 h at 4°C. Then the residual enzyme activities were measured at optimum pH and temperature.

Substrate specificity

Substrate specificity was determined by hydrolysis of different substrates like potato starch, amylose, corn starch, glycogen, and amylopectin in place of soluble starch under standard assay conditions (pH 6.5, 90°C). The relative values of the hydrolytic capacity of substrates were calculated on the basis of hydrolysis of soluble starch, considered as 100%.

Determination of blue loss percentage of starch

One mL soluble starch [1% (w/v) (Himedia, Mumbai, India) in phoshphate buffer, 10 mM, pH 6.5] was incubated with 0.1 mL of the enzyme at 90◦C for 30 min and enzymatic reaction was periodically stopped by adding 10 mL of 0.1 N HCl. The reaction mixture was diluted 10 times with iodine reagent [0.05% iodine (I_2) and 0.5% potassium iodide (KI)]. The drops of blue color of the starch iodine complex were determined colorimetrically at 660 nm [14].

Determination of anomeric configuration of hydrolyzed product

The anomeric configuration of hydrolysed products was determined by measuring the optical rotation. A reaction mixture (1 mL) contained of 1% (w/v) starch solution in 10 mM phosphate buffer (pH 6.5) and 0.1 mL of purified enzyme in a 1 cm cuvette. The optical rotation of the mixture was periodically measured in polarimeter (Perkin Elmer, USA) using sodium light. The mutarotation of the hydrolysate was determined by adding 5.0 mg of solid sodium carbonate per milliliter of reaction mixture after the optical rotation became almost constant [15].

Effect of inhibitors on enzyme activity

To investigate the effects of different inhibitors, the purified α-amylase was pre-incubated for 1 h with inhibitors like iodoacetamide, p-chloro mercuro benzoate in 10 mM concentration and ethylenediaminetetra acetic acid (EDTA) in 1.0–5.0 mM concentrations. The residual activity was determined under optimal assay conditions (pH 6.5 and 90°C) and relative activity was determined by considering the activity in absence of any additives as 100%.

Chromatographic analysis

The hydrolytic products were determined by digesting the starch (1% in 10 mM phosphate buffer, pH 6.5) with purified α–amylase at 90-92°C at different time intervals (10 to 180 min). The periodic hydrolytic products were detected by spotting the sample (100 μL) on 1.0 mm Whatman No.1 chromatography paper. A descending mode of solvent system of n-butanol-acetic acid-water

in 4:1:5 ratios (v/v) were used for separation. Chromatogram was developed by dipping the paper in alkaline silver nitrate/sodium hydroxide reagent (1.2%. $AgNO_3$ + 1.2% NaOH + 5% $Na_2S_2O_3$) and the hydrolyzed products were identified on the basis of maltooligosaccharide marker (Sigma,USA).

Results

Alpha-amylase of *Bacillus licheniformis* SKB4 was purified through a multi step process from the culture broth and the results of successive recovery of the enzyme are shown in Fig. 1, 2 and Table 1. The figure 1 showed that most of the fractions from DEAE cellulose column contained proteins, but amylase activity was present in the fraction between 7-16. The effluent of Sephadex G-100 column provided only one symmetrical protein peak associated with the amylase activity (Fig. 2). Finally the enzyme was purified about 214 fold with specific activity of 827 U/mg and 64.8% yield of recovery. The homogeneity of the enzyme was indicated by a single protein band on SDS-PAGE (Fig. 3). The estimated molecular weight of the enzyme was found to be 60 kd having optimum pH and temperature 6.5 and 90°C respectively [16].

Salt tolerance capacity of the purified amylase of *Bacillus licheniformis* SKB4 at various NaCl concentrations is shown in Fig. 4. The amylase retained 80% of its original activity at 5 M NaCl solution (Fig. 4). Substrate specificity is a general feature of every enzyme and the results of hydrolysis of various substrates were given in Fig. 5. Soluble starch is the best substrate (100% digestion) for α-amylase of *Bacillus licheniformis* SKB4 followed by others anylopectin (90%)>potato starch (86%)>corn starch (81%)>amylose (71%)> glycogen (44%). The purified amylase had shown a rapid loss of intensity of the blue color of starch-iodine complex (blue loss percentage) in Fig. 6. About 30% color disappeared within 10 minutes and after 30 minutes the blue loss of reaction mixture had been completed. The result of study of optical rotation revealed that degree of rotation had been decreased along with time and sudden downward fall of rotation had been observed at 60 minutes. The downwards shifting of optical rotation (Fig. 7) of starch hydrolytic products in respect to the time indicated that the products were in α-anomeric configuration. The enzyme activity was unaffected by metal ion chelator EDTA up to 1.0 mM concentration; then the relative activity was gradually reduced along with increasing concentration of EDTA and the activity was completely inhibited at 5.0 mM concentration (Fig. 8). The enzyme activity was not inhibited by β-specific inhibitors p-chloromercuro benzoate and iodoacetamide (10mM) (Fig. 9).

The end products of starch hydrolysis by purified α-amylase were analyzed by paper chromatography (Fig. 10). The result indicated that the α-amylase from *Bacillus licheniformis* SKB4 produced oligosaccharides such as maltotriose, maltotetrose, maltopentose from starch at an early stage of the reaction. As the reaction progressed these oligosaccharides were further hydrolyzed to smaller oligosaccharides and maltose but not glucose. This result indicated that the enzyme attacked the bonds randomLy in the inner region of polysaccharides and produced various maltooligosaccharides.

Table 1:- Summary of purification of α - amylase from culture broth of *Bacillus licheniformis* SKB4.

Purification steps	Volume (ml)	Total activity (Units)	Total protein (mg)	Specific activity (U $mg^{-1)}$	Purification fold	Yield (%)
Crude extract	200	3700	958	3.86	1	100
Salting out with 35-80% $(NH_4)_2SO_4$	4.00	3200	42.56	75.18	19.47	86.4
DEAE cellulose column chromatography	4.00	2640	22.0	120.0	31.08	71.3
Sphadex G-100 Gel filtration chromatography	4 .00	2400	2.9	827	214	64.8

Specific activity = Total activity / Total protein; Fold of purification = Specific activity in the respective step / Specific activity in the crude extract; Yield % = [Total activity in respective step/ Total activity in crude extract)] × 100.

Figure 1:- Chromatography of amylase on DEAE-cellulose column (2.5 x 30 cm), elution started with 10 mM phosphate buffer pH-6.5, followed by a salt gradient (KCl up to 0.1M) in same buffer. The enzyme was eluted at a flow rate of 1ml/min (2 ml/tube).

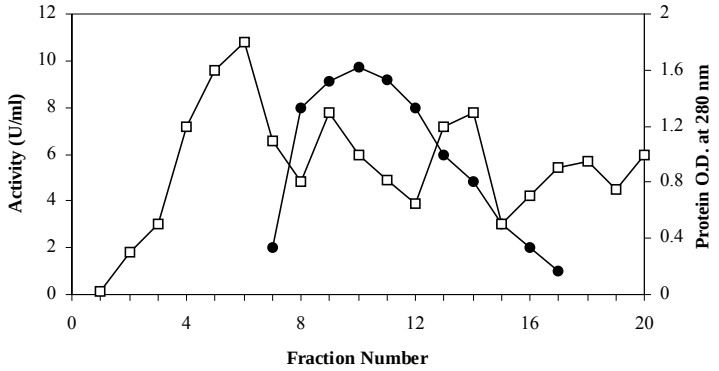


Figure 2:- Gel filtration of partially purified amylase of *Bacillus licheniformis* SKB4 on Sephadex G-100 column (1.5 x 92 cm). Elution was carried out by using 10 mM phosphate buffer, pH- 6.5 at flow rate of 0.5 ml/min (1 ml/tube).

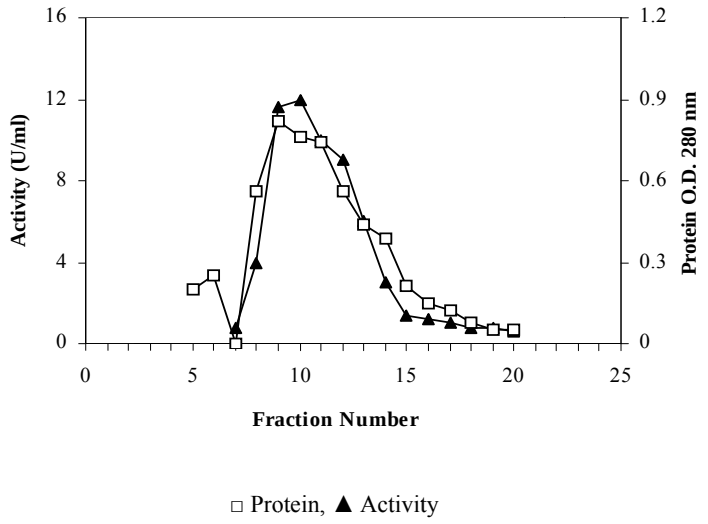


Fig 3:- Determination of molecular weight by SDS- PAGE. Lane 1st Molecular marker, 2nd after DEAE-cellulose chromatography, 3rd after gel filtration. Molecular Marker A-Myosin; 200 kd, B- β-glactosidase;116.2 kd, C- BSA; 66.7 kd, D- ovaalbumin; 45 kd, E- chymotrypsinogen;13.7 kd. α-amylase of 3rd lane is 60 kd. Lane 4- α-amylase from *Bacillus licheniformis* SKB4 were assumed by their starch hydrolyzing activity in presence of iodine (1.3%).

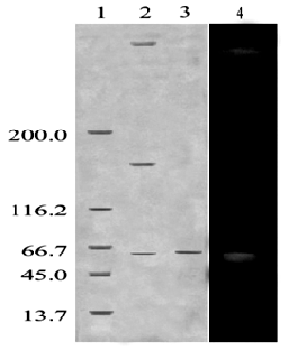


Fig 4:- Effect of NaCl concentration on amylase activity. Enzyme was incubated in 1 to 5 M NaCl concentration and residual enzyme activity was measured at pH 6.5 and 90°C respectively

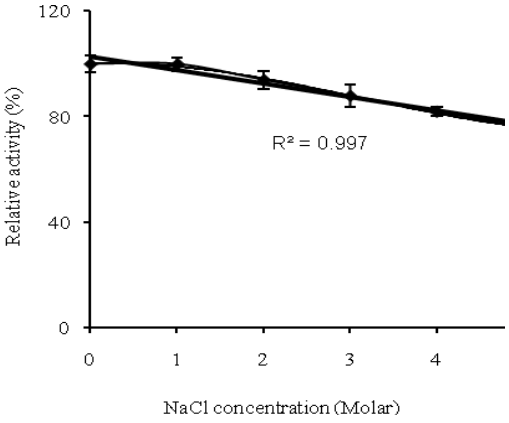


Fig 5:- Determination substrate specificity of amylase from *B. licheniformis* SKB4. Substrates :- 1- soluble starch, 2- potato starch, 3- amylose, 4- corn starch, 5- glycogen, 6- amylopectin**.**

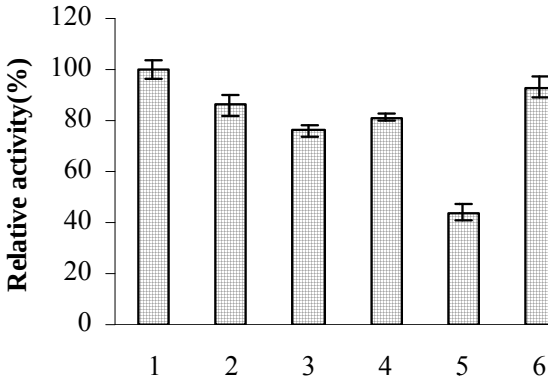


Figure 6:- Percentage loss of starch iodine colour complex in presence of starch as substrate after enzymatic degradation by amylase from *Bacillus licheniformis* SKB4. Sample were withdrawn at 0, 5, 10, 15, 20, 25, 30 minutes and processed for formation blue colour in presence of iodine

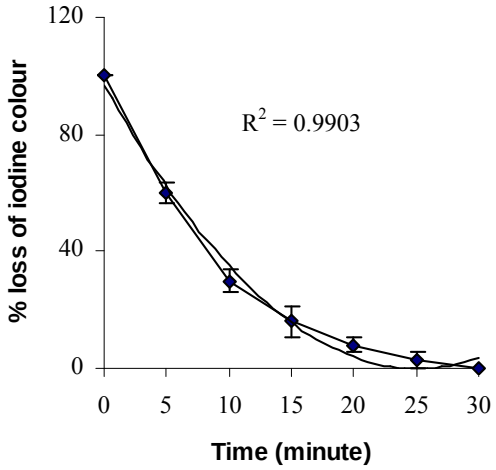


Figure 7:- Optical rotation of the products formed by the action of the purified amylase from *Bacillus licheniformis* SKB4 in presence of solid sodium carbonate (30mg).

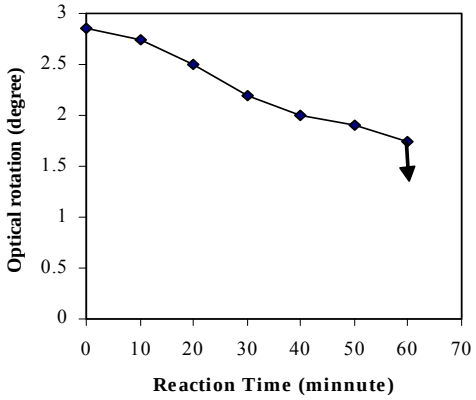


Figure 8:- Effect of EDTA at various concentration ons the activity of amylase of B. licheniformis SKB4. Amylase activity measured at pH 6.5 and 90°C.

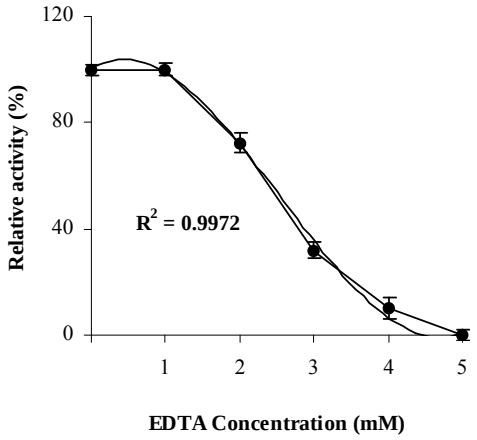


Figure 9:- Effect of enzyme inhibitors on amylase activity of *Bacillus licheniformis* SKB4. 1. Control (no inhibitor). 2. p-Chloromercuro benzoate (10 mM), 3. Iodoacetamide (10 mM). Amylase activity measured at pH 6.5 and 90°C.

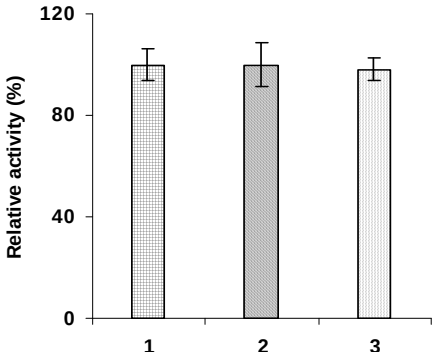
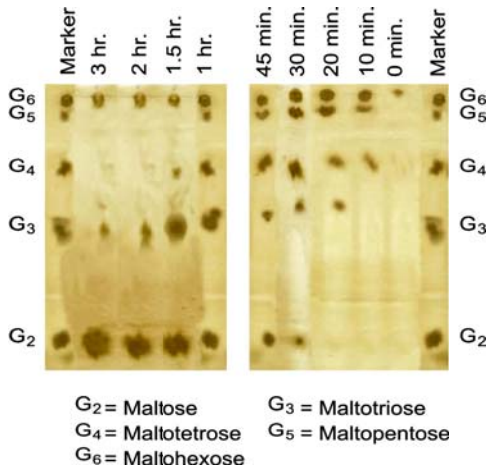


Figure 10:- Time course of the hydrolysis of starch by α-amylase from *Bacillus licheniformis* SKB4; analyzed by paper chromatography. The reaction mixture contained 1 % starch in 0.1 M phosphate buffer (pH 6.5) at 90-92°C and samples were taken at various time intervals. The chromatogram was prepared on I mm Whatman chromatographic paper. Solvent system was n-butanol-acetic acid-water 4:1:5 (upper phase). Spots were revealed using alkaline silver nitrate solution (1.2%) and 5% thiosulfate solution.



Discussion

The multi step process of alpha-amylase purification was very common with satisfactory yield value. The amylase from *Bacillus licheniformis* SKB4 was purified about 214 fold and 64.8% yield of recovery. This traditional multi step process of purification was adopted by several workers [17-20] and their yield of purification was much lower than the present study. Normally, the traditional methods give a lower yield but the results of purification of this enzyme were satisfactory (yield 64.8%) in comparison with an advanced method like chromatofocusing, HPLC technique [21,22]. In these multi step purification system, a considerable amount of enzyme is lost due to autolysis and some remain physically adsorbed on the matrix. To overcome these constraints, single step purification systems like affinity chromatography, counter current chromatography, expanded bed chromatography, etc., are now being employed, [23] but the homogeneity of the purified enzyme are less than conventional methods [22]. The observed value of the molecular weight of amylase of *Bacillus licheniformis* SKB4 on SDS-PAGE was 60 kd. Morgan and Priest [24] estimated the molecular weight of α-amylase from *Bacillus licheniformis* was 62.65 kd. Fogarty [25] argued that most of the α-amylases have a molecular weight of about

50 Kd. Prakash and Jaiswal [26] reported that thermostable α-amylase from *Bacillus licheniformis* is a monomer of ~58 kd exhibiting an α/β-barrel in its central part.

Salt tolerance capacity of the studied amylase was very high and remained active 5 M NaCl concentration. Earlier, Jana et al. [27] and Cordeiro et al. [28] reported that α-amylase from *Bacillus megaterium* and *Bacillus* sp. retain 80% and 47% of its original activity, respectively at 5 M NaCl solution after 24 h incubation at 4°C. This amylase was able to hydrolyze several types of starch in various percentages. Among them soluble starch had shown the best result followed by others. Similar type of observation was also reported by Jensen and Olsen [29]. Valaparla [30] reported that soluble starch is the best substrate of α-amylase from *Acremonium sporosulcatum.*

Rapid blue loss property had been observed during the reaction with starch-iodine complex. This observation indicated that the enzyme breaks α-1,4 glycosidic bonds of the substrate in a fashion of multiple attack system and produces small oligosaccharides with a chain length of below 18 glucose units because Bailey and Whelan [31] suggested that the minimum chain length necessary for iodine color formation is 18 glucose units. Bijttebier et al. [32] reported that amylase from *Bacillus stearothermophilus* showed a maximum degree of multiple attacks in respect to other sources. Rapid reduction of blue value and endolytic mode of action of α-amylase were also reported by Dheman et al. and Jana et al. [33,18]. Multiple attack on a substrate by different amylases was argued by several authors [34-37]. Akerberg et al. [38] opined that amylolysis of starch by the exo-attacking enzymes is accompanied by a relatively little reduction of the iodine staining capacity of the substrate due to sequential release of reducing sugars. The purified amylase produced α-anomeric products after hydrolysis starch. Similar findings of optical rotation were obtained by Anindyawati et al. and Jana et al. [39,18]. The amylase was not inhibited by low concentration of EDTA (1.0 mM). While, higher concentration of EDTA can able to inhibit enzyme activity. This inhibition of enzyme activity by EDTA may due to irreversible inactivation of the enzyme. Chelator resistant α-amylase is also reported by Kikani and Singh [17]. Earlier, Abdel-Fattah et al. [40] reported that the inhibitory effect of EDTA on α-amylase from *Bacillus licheniformis* is concentrations dependent. β-specific inhibitors p-chloromercuro benzoate and iodoacetamide (10mM) were unable to stop the enzyme activity. Addition of thiol group specific inhibitors, blocks the cysteine residue and inhibits the β-amylase activity. Haifeng et al. [41] reported that α-amylase is not inhibited by iodo-acetic acid. Generally, three amino acids like aspertate-206, glutamate-230, aspertate-297 [42**]** are present in the active site of the α-amylase, whereas, β-amylase is thiol group dependent and carries two cysteine residue at position of 95 and 343 at its active site [43]. The purified amylase showed rapid reduction of blue loss percentage of the reaction mixture (Fig 6), produced α-anomeric hydrolytic products from starch (Fig. 7) and activity was not affected by thiol group specific inhibitors (Fig 9), and low concentration of EDTA (Fig 8). All these findings indicated that the amylase of *Bacillus licheniformis* SKB4 belonged to endo-attacking, metal independent [16] α-amylase.

The α-amylase from *Bacillus licheniformis* SKB4 produced malto-oligosaccharides from starch at an early stage of hydrolytic reaction and later gave smaller oligosaccharides and maltose. This result indicated that the enzyme attacked the bonds randomLy in the inner region of

polysaccharides and produced various maltooligosaccharides. Alpha-amylase from the cyanobacterium *Nostoc* sp. PCC 7119 also produces maltose and maltooligosaccharides from soluble starch [44]. Previously, maltotriose, maltopentose forming amylase from different species of *Bacillus* was also reported [45, 18]. Xie et al. [19] reported that α-amylase from *Bacilus* sp. IMD 434 initially hydrolyzed starch to maltose and maltotriose, which was further hydrolyzed to maltose and glucose.

Conclusion

The α-amylase of *Bacillus licheniformis* SKB4 has some special characteristics. The enzyme was purified up to its homogeneity through a low-cost, simple chromatography process with 64% yield value. It had some unique properties like high thermostability, salt tolerance capacity, metal ions independence and maltooligomer producing ability. These features make the enzyme a promising commercial exploitation, especially in food, pharmaceutical industries. In the future, genetic manipulation of this organism will be essential to meet the industrial challenge.

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Conflict Of Interest

The authors declare that there are no conflicts of interest.

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