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Full Length Article

Production, Optimization and Partial Characterization of Thermostable and Alkaline Amylase from *Bacillus licheniformis* KSU-6

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Abstract

Total of 56 bacterial isolates were isolated from five palm tree cultivated soil samples and screened their amylase production. In the preliminary screening, twenty four isolates exhibited amylase producing ability. Among these 24 isolates, an isolate KSU-6 showed maximum amylase enzyme production (37.52 U mL^{-1}), and the isolate was identified as *Bacillus licheniformis* KSU-6. The maximum amylase production was optimized, and the conditions were pH 10.0, temperature 50ºC, incubation period 44 h, inoculum concentration 2% (v/v), starch 0.2% (w/v) as carbon source, peptone 0.5% (w/v) along with malt extract 0.3% (w/v) as nitrogen sources for *B. licheniformis* KSU-6. The partially purified amylase stabled upto pH 12, temperature 80ºC, 1-2 M salt concentration and 10 mM of different detergents. In this study conclude that the soil bacterium *Bacillus licheniformis* KSU-6 produced thermostable and alkaline amylase, which could be useful for industrial liquefaction and detergent industries. © 2016 Friends Science Publishers

Keywords: Alkaline; Amylase; *B. licheniformis*; Thermostable; 16S rRNA

Introduction

The enzymes have great applications in industrial and house hold products as they are eco-friendly in nature. Among the enzymes, amylase alone only contribute approximately 25% of the industrial world enzymes market (Reddy *et al.,* 2003), and which have been used in industrial sectors including brewing, baking, dairy, textile, laundry, paper, and also useful in the fine chemical and pharmaceutical industries (Gupta *et al*., 2003; Schallmey *et al.,* 2004; Das *et al.,* 2011). Many researchers have been previously reported different types of extracellular enzymes produced by the genus *Bacillus*. Of them, amylase and protease are very important enzymes in industrial level (Burhan *et al.,* 2003; Dhanasekaran *et al.,* 2006; Saxena *et al.,* 2007; Alamri, 2010; Vaseekaran *et al.,* 2010; Divakaran *et al.,* 2011; Abdel-Fattah *et al.,* 2013; Deb *et al.,* 2013). Amylase plays a vital role in the hydrolysis of starch to dextrin and other small polymers constituted of glucose units (Windish and Mhatre, 1965), and the enzyme derived from various sources including plants, animals and also microorganisms (Pandey *et al.,* 2000).

Even though its extensive applications in industries, amylases obtained from mesophilic organisms failed to cope up with the industrial processes that are carried out in the presence of salt solutions, organic solvents, heavy metals, high temperature and extremes of pH that would be inhibit the enzymatic activity (Saxena *et al*., 2007). Hence, there arises a need for identifying amylases that are functional under such extreme conditions including high pH, temperature and organic solvents. The thermostable and alkaline amylase, which could be used for saccharification process in starch, textile industries and also ingredient with detergents for laundries (Bragger *et al*., 1989; Kim *et al.,* 1995; Saxena *et al.,* 2007). In Saudi Arabia more than 90% of the land areas are deserts but the microbial diversity in this habitat and the potential industrial enzymes from them have been only very poorly explored (Abu-Zinada *et al.,* 1981; Al-Shehri and Mostafa, 2004; Alamri, 2010; Alrumman *et al*., 2014). Hence, we took to screening of the palm tree cultivated soil for bacteria with special reference to amylase enzyme production. Furthermore, the fermentation conditions were optimized for the maximum amylase production by *Bacillus licheniformis* KSU-6, and characterized the partially purified amylase.

Materials and Methods

Isolation and Screening of Amylase Enzyme Producer

Soil samples were collected from five different Palm tree cultivated area in Riyadh Province of Saudi Arabia.

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The samples were serially diluted in sterile saline water and the dilutions (10^4 and 10^5) were plated in nutrient agar plates and incubated at 37ºC for 48 h. Based on the morphology, size and colour, the colonies were picked and purified in nutrient agar. All the isolates were subjected to screening their ability of amylase enzyme production using starch agar plates (10 g/L soluble starch, 4 g/L yeast extract, 1 g/L K_2HPO_4 and 1.5 g/L $MgSO_4.7H_2O$, pH 7). The isolates were streaked on starch agar plates and incubated at 37ºC for 24 h. The plates were flooded with Gram's iodine solution, and the isolates were selected based on the clear zone formation, and categorized as excellent, good, moderate and low enzyme producers.

Amylase Production and Enzyme Assay

The isolates showing significant amylase activity (zone of clearance >15 mm) were inoculated in enzyme production medium (2 g/L starch, 5 g/L peptone and 3 g/L beef extract, pH 7) and incubate at 37ºC under 120 rpm shaking condition for 24 h. The supernatants were obtained by centrifugation at 10,000 rpm for 10 minutes at 4ºC in refrigerated centrifuge (Eppendoff, Germany) and the supernatants were used for amylase assay (Hagihara, 1958). One millilitre of enzyme (supernatant) was taken and 1 mL of soluble starch (1% w/v in 0.1 M sodium phosphate buffer, pH 7.0) was added and incubated the mixture at 45ºC for 15 mins. The reaction was terminated by adding 2 mL of 3,5 di-nitro salicylic acid (DNSA) and incubated at 90ºC for 10 mins. The activity of amylase enzyme was estimated by reading the optical density by using spectrophotometer at 540 nm. The activity of amylase enzyme was discriminated by International Unit, and one unit of enzyme was defined as the amount of enzyme releasing 1 µmol of reducing sugars per minute under standard assay conditions. The isolate KSU-6 was showed maximum amylase production therefore the isolate KSU-6 was selected for further studies.

Identification of Isolate KSU-6

The bacterial isolate KSU-6 was identified by various biochemical tests and also 16S rRNA gene sequence analysis. Genomic DNA was extracted from the isolate KSU-6 using MN DNA extraction kit (Germany) according to the manufactures protocol. The culture broth (2 mL) of the isolate KSU-6 was taken and centrifuged at 8,000 x g for 5 min. The supernatant was discarded and the pellet was re-suspended in 200 µL of buffer B3 and 25 µL of Proteinase K were added and incubated at 56ºC for 1 h. After that 210 µL of ethanol (96%) was added and vortexed vigorously then transferred to NucleoSpin tissue column placed over a collection tube and centrifuged for 1 min at 11,000 x g. 500 µL of wash Buffer (BW) was added and centrifuged at 11,000 x g for 1 min. Wash buffer (B5), 600 µL, was added and centrifuged. The flow-through was discarded. The NucloSpin tissue column was placed in a 1.5 mL micro-centrifuge tube, 100 µL of pre-warmed elution buffer (BE – 70ºC) was added, incubated at room temperature for 1 min, centrifuged and the DNA was obtained. The extracted DNA was amplified by using the universal bacterial 16S rRNA primers such as forward primer 8F 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer 1492R 5' TACGGTTACCTTGTTACGACTT 3'. The amplified PCR products were purified and sequenced in Macrogen (South Korea). The sequences were compared with other bacterial sequences by BLASTn. The gene sequences of the isolate KSU-6 was submitted in Genbank and got the accession number. Phylogenetic tree was constructed based on maximum similarity of 16S rRNA genes sequences which has already published in Genbank using MEGA v6.0 (Tamura *et al*., 2013).

Optimization of Amylase Production

The following parameters such as pH, temperature, incubation period, inoculum size, carbon and nitrogen sources were assessed for maximum amylase production. For the effect of pH, the media was adjusted to pH 5, 6, 7, 8, 9, 10, 11 and 12 using 1N NaOH and 1N HCl and *B. licheniformis* KSU-6 was inoculate and incubated at 37ºC for 24 h and amylase enzyme production was determined. The isolate KSU-6 was inoculated and incubated at the temperature ranges from 25 to 60ºC at 5ºC interval then the amylase enzyme production was assessed. The isolate KSU-6 was inoculated in the production medium and incubated at 40ºC for 24 to 52 h. Samples were collected aseptically from flask in every 4 h interval. The effect of inoculum size 0.5, 1, 2, 4, 6, 8 and 10% also studied for maximum amylase production. The production media supplemented with different carbon sources including glucose, fructose, lactose, maltose, sucrose and xylose (0.2% w/v by replacing starch) and also nitrogen sources including casein, malt extract, meat extract, yeast extract, potassium nitrate and sodium nitrate (0.5 and 0.3% w/v by replacing peptone and beef extract, respectively) were also studied. The culture supernatant of the isolate KUS-6 was collected in each experiment and amylase assay was performed.

Partial Purification of Enzyme

Two litre of culture was centrifuged and the supernatant was used for purification of enzyme. The supernatant was precipitated using ammonium sulphate (85% saturation). The precipitate was dissolved in glycine NaOH buffer (0.05 M, pH 10) and dialysed overnight using the same buffer solution (Saxena *et al.*, 2007). The partially purified enzyme was used for characterization studies.

Effect of Physical and Chemical Sources on the Stability of Partially Purified Amylase from *B. licheniformis* KSU-6

The partially purified enzyme was studied their pH stability by pre-incubating the enzyme (0.08 mg/ mL) in buffers

containing different pH values ranged from 4 to 12 at 37ºC for 1 h. The enzyme activities were quantified under standard assay conditions. The thermal stability of enzyme was determined by pre-incubating the enzyme (0.08 mg/mL) at different temperature from 40 to 100ºC for 1 h. Aliquots were withdrawn and the residual activity was measured by using amylase assay. For halostability study, the enzyme was pre-incubated with NaCl (0-4 M) at 37ºC for 1 h and the enzyme activity was calculated. The effect of various detergents on enzyme stability, the enzyme was pre-incubated with different detergent such as SDS (sodium dodecyl sulphate), CTAB (cetyl trimethylamonium bromide), Triton X-100, Tween 20 and Tween 80 at a final concentration of 10 mM individually at 37ºC for 30 min. The enzyme activities were determined and compared without detergents as a control.

Statistical Analysis

All the experimental data were analysed and expressed as means ± standard deviation (SD). Univariate Analysis of Variance (ANOVA) was performed on the data of pH, temperature, incubation periods, inoculum size, carbon and nitrogen sources along with amylase activity were determine the level of significance.

Results

Isolation, Screening and Identification of Amylase Producing Bacteria

Total of 56 bacterial isolates were obtained from five different palm tree cultivated soil samples. Altogether, only twenty four isolates had the potential of amylase enzyme production, whereas the remaining isolates not showed the clear zone formation. Among them, an isolate KSU-6 showed maximum amylase activity (37.52 U mL^{-1}) when compared to other isolates (Fig. 1). Hence, the isolate KSU-6 was selected for characterization and optimization studies. The Fig. 2 showed the morphology of the isolate KSU-6 in nutrient agar plate and amylase producing ability in starch agar plate. The isolate KSU-6 belongs to Gram-positive, rod-shaped, catalase positive and oxidase negative bacterium. Based on the morphological, physiological and biochemical characterization, the isolate KSU-6 showed up to closely related the genus *Bacillus*. The 16S ribosomal RNA gene was amplified from the genomic DNA of the isolate KSU-6 (Fig. 3a). The amplified product was sequenced and submitted in GenBank under the accession number KP229436. The BLAST result of the isolate was found 100% sequence similarity with *B. licheniformis* strain CCMMB931 (KF879274). On the other hand, the phylogenetic relationship of the isolate KSU-6 was formed separate cluster along with *B. licheniformis* strain B37 (JX105529) (Fig. 3b). On the whole, physiological, biochemical and molecular characterization, the isolate was identified as *B. licheniformis* KSU-6.

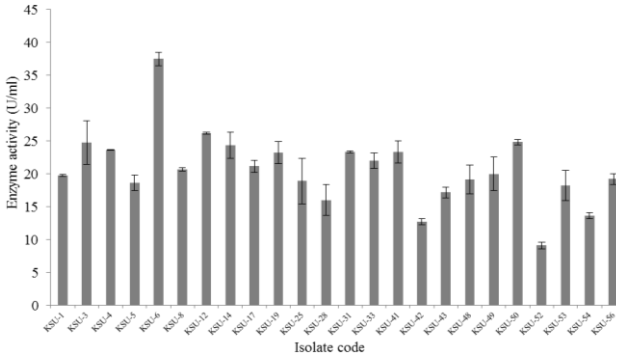

Fig. 1: Qualitative detection of amylase activity by soil bacterial isolates

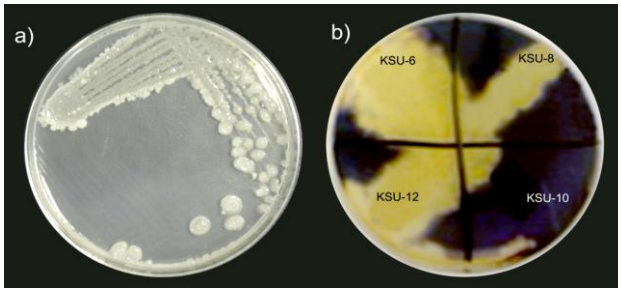

Fig. 2: a - Colony morphology of *Bacillus licheniformis* KSU-6 grown on nutrient agar medium; b – Amylase activity in starch agar plate

Optimization of Amylase Enzyme Production

Effect of pH: The effect of different pH of medium revealed that *B. licheniformis* KSU-6 was able to grow and also produced extracellular amylase over wide range of pH. The enzyme activity at the initial pH 6 was 29.76 U mL^{-1}, whereas the medium pH was increased, the amylase enzyme production was also increased linearly up to pH 10. Maximum amylase production was obtained (41.24 U mL^{-1}) at pH 10 as presented in Fig. 4a. The results of statistical analysis between the pH and enzyme production showed the level of significance $p = 0.05$.

Effect of temperature: The incubation temperature was optimized for maximum amylase production for *B. licheniformis* KSU-6. The maximum enzyme production was obtained (45.21 U ml^{-1}) at 50ºC followed by 39.32, 38.78, 32.02, 25.35, 23.32, 19.03 and 16.91 U mL^{-1} for 45, 55, 40, 60, 35, 30 and 25ºC respectively (Fig. 4b). The results are statistically significant at $p < 0.05$.

Effect of incubation period: Amylase enzyme production with different incubation periods was assessed for *B. licheniformis* KSU-6. The maximum amylase production was achieved (47.68 U mL^{-1}) after 44 h of incubation (Fig. 4c). On the other hand, the enzyme production was declined (45.26 U mL^{-1}) after 48 h of incubation period due to the static phase of growth. The results are statistically not significant.

Table 1: Effect of pH, temperature, NaCl and detergents on the stability of partially purified amylase from *Bacillus licheniformis* KSU-6

Physical, chemical sources and stability of amylase (%)							
pH	REA	Tem.	REA	NaCl	REA	Detergents (10 mM)	REA
4	45	40	73	0 M	100	Control	100
5	63	50	100	1 M	100	SDS	91
6	88	60	100	2 M	100	CTAB	79
7	92	70	97	3 M	88	Triton X-100	88
8	100	80	89	4 M	73	Tween 20	79
9	100	90	65			Tween 80	85
10	100	100	58				
11	91						
12	85						

Relative activity of the partially purified amylase was calculated from the original activity as 100 % (156 U/ml at pH 8 and 50 ºC); REA, Relative Enzyme Activity (%); Temp., temperature. The statistical analysis revealed the significant difference (p =0.05) between the concentrations of pH, NaCl, detergents and their relative enzyme activities

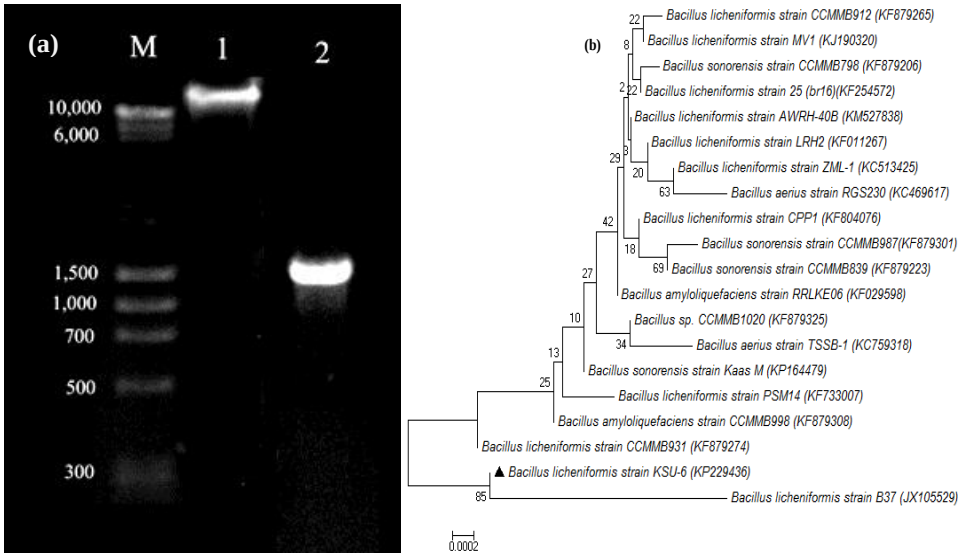

Fig. 3: a – Molecular analysis of *B. licheniformis* KSU-6; b - Phylogenetic tree based on 16S rRNA gene sequences *B. licheniformis* KSU-6 and other colsely related *Bacillus* spp
M, molecular marker; 1, Genomic DNA of KSU-6; 2, 16S rRNA of amplified product

Effect of inoculum concentration: The inoculum size plays an important role in amylase production. Totally seven different inoculum concentrations were employed, among them, 2% (v/v) of inoculum was suitable for maximum amylase enzyme production for *B. licheniformis* KSU-6 and the enzyme production was achieved 48.25 U mL^{-1} (Fig. 4d). The concentration of inoculum was increased from 2% (v/v), the enzyme production showed a declining trend. The lowest enzyme activity 15.33 U mL^{-1} was obtained at 10% of inoculum concentration. The results are statistically significant at $p < 0.01$ was observed between the concentration of inoculum and amylase enzyme production.

Effect of carbon sources: The effect of carbon sources on amylase production for *B. licheniformsis* KSU-6 was determined. Among the different carbon sources tested, 0.2% starch (w/v) was found to be a good carbon source for amylase production (52.43 U mL^{-1}), followed by maltose (33.52 U mL^{-1}), glucose (28.73 U mL^{-1}), fructose (26.89 U mL^{-1}), xylose (23.56 U mL^{-1}), lactose (23.37 U mL^{-1}) and sucrose (19.21 U mL^{-1}) (Fig. 4e).

Effect of nitrogen sources: The nitrogen sources are one of the important nutrients for amylase enzyme production. Among the different nitrogen sources tested, beef extract concentration constant along with 0.5% (w/v) of peptone exhibited maximum amylase production as 47.43 U mL^{-1}. On the other hand, peptone concentration was constant and supplemented with different nitrogen sources, the maximum amylase production (56.32 U mL^{-1}) was achieved with 0.3% (w/v) malt extract for *B. licheniformis* KSU-6 and the results were presented in Fig. 4f.

Stability of Partially Purified Amylase from *B. licheniformis* KSU-6

The partially purified amylase was characterized based on the stability of pH, temperature, NaCl and detergents,

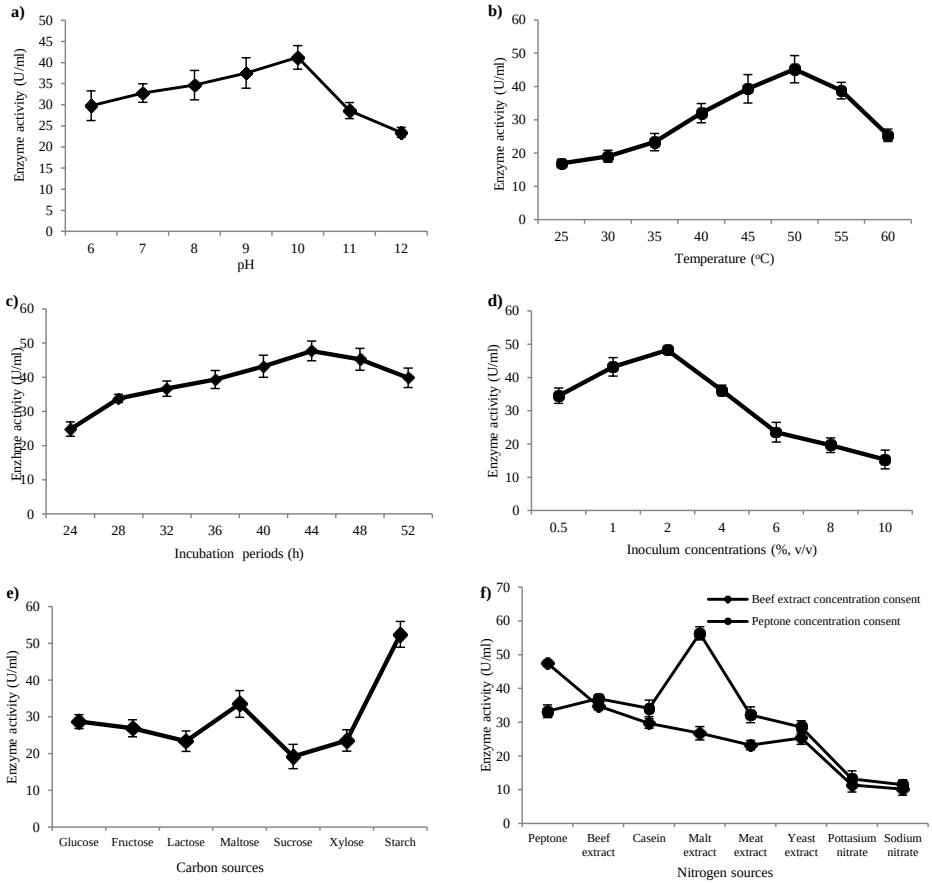

Fig. 4: a - Effect of pH; b - temperature; c - incubation periods; d - inoculum concentrations; e - carbon sources; f - nitrogen sources on amylase enzyme production

Values represent the means of 3 samples and error bars denote to standard deviation of the value; the statistical analysis revealed the significant difference between the enzyme production and pH (p =0.05), temperature (p<0.05), inoculum concentration (p <0.01)

and the results are presented in Table 1. The pH stability of amylase was determined and the enzyme was active wide range of pH from 4.0 to 12.0. The relative activity 100% was observed at pH 8, 9 and 10, whereas 91 and 85% of relative activities were obtained at pH 11 and 12, respectively. Thermal stability profile of amylase, the enzyme was pre-incubated at various temperatures for 60 min and the enzyme activity was assayed. The results revealed that the highest stability (100%) was observed at 50 and 60ºC, but the enzyme retained 58% after heating at 100ºC for 60 min. Similarly, the enzyme exhibited 100% of the stability in the presence of 0-2 M NaCl, and further the enzyme retained 73% of stability in 4 M NaCl. On the other hand, 91% relative activity was observed when the enzyme pre-incubated at 37ºC for 30 min in 10 mM sodium dodecyl sulphate followed by Triton X-100 (88%), Tween 80 (85%), CTAB (79%) and Tween 20 (79%).

Discussion

In bacteria, the genus *Bacillus* produces a large variety of extracellular enzymes, in which amylases are mainly considerable industrial importance (Swain *et al.*, 2006). In this study, total of 56 bacterial isolates were isolated from palm tree cultivated soil, of which an isolate KSU-6 was selected on the basis of level of amylase production and identified as *B. licheniformis* KSU-6 based on various morphological, biochemical and 16S rRNA gene sequence analysis. The enzyme production is often influenced by the medium components, fermentation conditions including pH, temperature, salinity, inoculum size, incubation periods, and nutrients such as carbon and nitrogen sources (Kundu *et al.*, 1973; Gupta *et al*., 2003; Saxena *et al*., 2007; Deb *et al*., 2013). In the present investigation, the required conditions have been optimized for enhancing the amylase enzyme production by *B. licheniformis* KSU-6. The physical parameters of the medium play an important role in the production of enzyme. In this study the minimum enzyme production was recorded at initial pH 6 and maximum amylase production was obtained at pH 10. The results indicated that there is a stimulation of enzyme production in the alkaline pH. Similarly, Saxena *et al.* (2007) reported that

the maximum amylase production was achieved at pH 10 for *Bacillus* sp. PN5 and also pH 5 to 12 supported for amylase enzyme production. Another study also conducted by Deb *et al.* (2013) stated that the maximum alpha amylase production was obtained by *B. amyloliquefaciens* P-001 at pH 9.0.

Amylases are produced at wide range of temperature (35-80ºC) have been previously reported from *B. amyloliquefaciens, B. licheniformis, B. stearothermophilis, B. subtilis* and *B. stearothermophilus* (Mielenze, 1983; Syu and Chen, 1997; Mishra *et al*., 2014; Saxena *et al*., 2007; Deb *et al*., 2013). In this study, the enzyme production was carried out at different temperature, and the maximum enzyme production was obtained at 50ºC. While the temperature was increased or decreased from the optimum level the enzyme production was also declined. Saxena *et al*. (2007) reported *Bacillus* sp. PN5 capable of producing amylase from 30-70ºC and maximum amylase production at 60ºC. Lin *et al.* (1998) have also been reported 55ºC favoured for amylase enzyme production from *Bacillus* sp. TS-23. It is revealed that *B. licheniformis* KSU-6 grew well in the media and produced enzyme up to 60ºC, and it is proved that the isolate producing enzyme having thermotolerant ability and it can be classified as moderately thermostable amylase.

Amylase enzyme production was also assessed in different incubation periods for *B. licheniformis* KSU-6, and the maximum amylase production was achieved after 44 h. The enzyme activity was obviously declined by further increasing the incubation period from the optimum level. *Bacillus* sp. AMRI-01 was ability to produced maximum amylase enzyme after 48 h has been reported by Alamri (2010). The maximum amylase production was achieved from *B. licheniformis* Shahed-07 and *B. megaterium* have already been reported at 26 and 48 h incubation period respectively (Rasooli *et al.,* 2008; Oyeleke *et al.,* 2010). Lin *et al*. (1998) stated that the inoculum concentration is one of the important factors to be considered while optimizing the enzyme production. The effect of different inoculum sizes on amylase production was determined, 2% (v/v) level of inoculum was suitable for maximum amylase production for *B. licheniformis* KSU-6. Similarly, Mohanasrinivasan *et al*. (2014) have been reported that 2% of inoculum density was favourable for maximum amylase production for *B. pumilus* and also Mishra *et al*. (2014) reported that 1.5% of inoculum concentration favoured the maximum amylase enzyme production in *Bacillus* sp.

In fermentation media, the nature and amount of carbon sources are influenced in amylase production for the bacterial isolates. In this study, the maximum amylase production was observed 0.2% starch as sole source of carbon when compared to other carbon sources tested for *B. licheniformis* KSU-6. These results are supported with the previous reports stated that the amylase production was increased when starch used as carbon sources (Lachmund and Ruttkowski, 1990; Lin *et al*., 1998; Gupta *et al.,* 2003; Saxena *et al*., 2007; Bozic *et al.,* 2011). The nitrogen sources have influenced the extracellular amylase enzyme production and also supplement with specific nitrogen source on enzyme production differs from organism to organism (Hillier *et al.,* 1996). Altogether, the various nitrogen sources tested, beef extract concentration constant along with 0.5% of peptone (w/v) showed maximum amylase production by *B. licheniformis* KSU-6. On the other hand, peptone concentration was constant and in addition with different nitrogen sources tested for amylase production by *B. licheniformis* KSU-6, the maximum enzyme production was achieved with 0.3% malt extract. Conversely, Teodoro and Martins (2000) and Saxena *et al*. (2007) have been reported that peptone along with yeast extract influenced the amylase production in *Bacillus* sp.

Amylase enzyme produced by *B. licheniformis* KSU-6 was precipitated and partially purified, which was used for characterization studies. The enzyme was pre-incubated with buffer containing different pH values and the amylase activity was assayed. The amylase retained 100% stability at pH 8-10, but there was a noticeable debility in the enzyme activity in acidic pH. Lin *et al.* (1998) and Saxena *et al.,* (2007) have been reported previously the amylase from *Bacillus* sp. that exhibited high stability in the range of pH 6.0 -13.0. However, the effect of temperature on enzyme stability was also assessed and the results showed that amylase produced by *B. licheniformis* KSU-6 retained the stability (58%) up to 100ºC. The present investigation results are strongly agreed with the previous report of Mamo *et al*. (1999) and stated 75-80ºC has the optimal temperature for amylase produced by *Bacillus* sp. The halostability of amylase showed that the enzyme was active at 0-4 M salt concentrations. Prakash *et al*. (2009) reported that the amylases from *Chromohalobacter* sp. has highly active in broad range of salt concentrations (0-25%). The enzyme used in detergent industries would be stable to various detergent ingredients. The present investigation, we have used SDS, CTAB, Triton X-100, Tween 20 and Tween 80 for stability of amylase enzyme derived from the isolate KSU-6. The maximum relative activity (91%) was observed when 10mM sodium dodecyl sulphate pre-incubated at 37ºC for 30 min. On the other hand, the relative activities 88, 85, 79% were observed for Triton X-100, Tween 80 and Tween 20, respectively. Saxena *et al.* (2007) have been reported that the amylase from *Bacillus* sp. PN5 has exhibit more than 80% relative activity when incubated with sodium perborate and sodium dodecyl sulphate.

Conclusion

A potential soil bacterium *B. licheniformis* KSU-6 produced substantial quantity of amylase enzyme. In this study, the cultural conditions and media constituents have been optimized for maximal amylase production. The enzyme capable to withstand the temperature up to 100ºC, pH up to 12, broad range of salt concentrations and various detergent

ingredients, thus, it is clearly indicate that the extracellular thermostable and alkaline amylase produced by *B. licheniformis* KSU-6, which could be useful for starch liquefaction and detergent industries.

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