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PRODUCTION OPTIMIZATION OF A-AMYLASE FROM *BACILLUS LICHENIFORMIS*

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Abstract: -

Amylases are of great significance in present day biotechnology. They constitute 25% of the industrial market. To meet the industrial demand there is a need of production optimization of α-amylase from the microbial source. In the present study, an attempt was made to isolate thermophilic bacterial strain producing thermophilic and alkaliphilic α-amylase. Among 23 isolates, isolate K7 gave maximum production of αamylase, which was later identified as Bacillus licheniformis. The organism gave maximum production of αamylase in medium containing g/l (w/v) beef extract 3.0, peptone 5.0 and starch 1.0%. Starch (1.75%, w/v) and peptone (0.15%, w/v) were optimized to be best carbon and nitrogen sources for α-amylase production from Bacillus licheniformis. Optimum production of the enzyme was observed when inoculated medium of pH 8.0 was incubated at 50°C for 48 h of incubation time.Further during optimization of reaction conditions, the enzyme gave maximum activity with 0.1 M Tris HCl buffer of pH 8.0 when incubated at a reaction temperature of 50°C for 10 min of incubation time. The enzyme showed high affinity towards starch (0.15%, w/v) as substrate. The thermophilic and alkaliphilic nature of the enzyme suggest its potential application in starch, detergent and textile industries.

Keywords*: - Starch, α-amylase, optimization, thermophilic, alkaliphilic.*

INTRODUCTION

Thermophilic organisms can produce unique biocatalysts under extreme conditions [1]. The enzymes produced by thermophilic organisms are usually thermostable in nature [2, 3]. In starch industries the most widely used thermostable enzyme is amylase [4]. Amylases account for 65% of enzyme market in world [5]. They hydrolyze α-1,4-glycosidic linkage in starch in an endo-fashion [6]. Amylases are ubiquitous in nature and can be produced by plants, animals and microbes [7, 8]. However microbial amylases have dominated industrial applications since they are more stable, economical and easily available [9]. Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production for industrial needs [10].

To meet the demand of industries low-cost medium is required for the production of α-amylase. Commercial production of α-amylase usually happen using submerged fermentations [11]. Optimization of physical and chemical parameters plays a significant role to enhance the production of enzyme [12, 13, 14].

Starch industries such as brewing and sugar production needs α-amylases that are active even at higher temperatures for gelatinization and liquefaction of starch to economize processes [15]. However other industries like textile and detergent industries require amylases working at alkaline pH-range. But most of the amylases from microbial sources have an optimum pH range 5.0-7.0 [16]. Hence alkaliphilic as well as thermostable amylases have gained a great attention now-a-days to meet industrial needs. Therefore in the present study an attempt was made to isolate a thermophilic organism producing thermostable as well as alkaliphilic α-amylase. The production conditions were optimized to get maximum yield of the α-amylase from the isolate. Further the reaction conditions were optimized to get maximum activity of the enzyme.

Materials and Methods

Isolation and screening of amylase producing thermophilic bacterial isolate from different sources

Water and soil samples were collected from different hot springs of Himachal Pradesh such as Tattapani in Shimla district, Kalath and Vashist in Kullu district in sterile centrifuge tubes and kept at 4°C in refrigerator till further processing. Enrichment was carried out in minimal salt medium containing strarch and incubated at 50°C for 24-48 h under shaking (120 rpm). After enrichment, 1 ml of the culture from enrichment was serially diluted to 10^{-4}-10^{-6} times with saline and was spread on nutrient agar plates and incubated for 24 h at 50°C. The isolates were sub-cultured once a month. For screening of amylase producing bacterial isolate, starch agar plates having growth of bacteria were flooded with Gram's iodine solution. A clear zone formation around the bacterial colonies was the indication of starch utilization. Further amylase activity of all primary screened isolates was checked by method given by Sengupta *et al*. (2000) [17].

Amylase activity

Amylase assay was performed by spectrophotometeric method described by Sengupta *et al*. (2000) [17], using starch as substrate and DNS as coupling reagent. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmole of glucose per minute under standard assay conditions.

Characterization of bacterial isolate

The bacterial culture was Gram stained and endospore staining was also done for morphological characterization of the organism. Further, the selected isolate was dentified by 16S rDNA sequencing at Xcelris Labs Limited, Ahmedabad.

Growth profile of *Bacillus licheniformis*

Growth profile of *B. licheniformis* was studied by evaluating biomass growth of selected bacterial isolate in starch seed medium. After every 6 h of incubation, optical density of culture broth was taken at 600 nm. The culture broth was then centrifuged at 12,000 rpm for 10 min and supernatant was used to assay enzyme activity.

Optimization Of ,Production Conditions For Maximum Production Of A-Amylase From *Bacillus Licheniformis*

Various physical and chemical factors have been known to affect the production of α-amylase. Although, optimum conditions may vary for each organism and enzyme certain factors have been established as the most significant in influencing overall enzyme yield.

Optimization of medium for the production of α-amylase from *Bacillus licheniformis*

Bacillus licheniformis was grown in 17 different media reported by previous workers. Medium-1 [18], Medium-2 [19], Medium-3 [20], Medium-4 [21], Medium-5 [22], Medium-6 [23], Medium-7 [24], Medium-8 [25], Medium-9 [26], Medium-10 [27], Medium-11 [28], Medium-12 [29], Medium-13 [30], Medium-14 [31], Medium-15 [32], Medium-16 [33], Medium-17 [34]. Each medium was used to produce α-amylase at 50±1°C and pH 7.0. All media were prepared in distilled water.

Optimization of carbon source and concentration of carbon source

Various carbon sources (starch, wheat flour, maize flour, arrow root, sodium citrate, sodium acetate, sucrose and glycerine, 1% w/v) were used. Different concentrations of optimized C-source (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 and 2.25% (w/v)) were used and the culture supernatant was assayed for enzyme activity.

Optimization of nitrogen source and concentration of nitrogen source

A concentration of 0.1% w/v of malt extract, beef extract, yeast extract, peptone, casein, ammonium sulphate, ammonium nitrate and urea were added to the production medium. Different concentrations (0.075, 0.1, 0.125, 0.15, 0.175 and 0.2%

(w/v)) of optimized nitrogen source were used in the production medium and the culture supernatant was assayed for enzyme activity. **Effect of metal ions on the production of α-amylase**
The production of amylase was studied individually in the presence of preselected metal ions (Mg^{+}, Na^{+}, Pb^{+}, Co^{2+}, Hg^{2+}, Fe^{3+}, Ca^{2+}, Cu^{2+}, K^{+}, and Zn^{2+}, 0.01% w/v each) that were separately included in the above optimized medium broth at pH 7.0.

Optimization of production temperature, pH and incubation time
For optimization of production temperature, the production medium was incubated at different temperatures viz. 35°C, 40°C, 45°C, 50ºC, 55ºC, 60ºC and 65ºC. The production medium of varying pH viz. 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 was inoculated with the culture and incubated in the rotary shaker for 24 h at 50°C and amylase activity was determined. And for optimization of incubation time the production medium was incubated in the shaker at 50°C of incubation temperature for the time intervals of 12, 24, 36, 48, 60, 72, 84 and 96 h and supernatant was assayed for enzyme activity.

Optimization of inoculum age and inoculum size
To optimize the inoculum age, seed culture was incubated for varying time *viz*., 6, 12, 18, 24, 30, 36 and 42 h the culture supernatant was assayed for enzyme activity. Inoculum size was optimized by inoculating the production medium with varying size of inoculum (1%, 2%, 3%, 4%, 5% and 6%, v/v) and assaying the supernatant for enzyme activity.

Optimization of reaction conditions for α-amylase
Various buffers screened were Citrate buffer (pH 4.0–8.0), Sodium phosphate buffer (pH 5.0-9.0), Potassium phosphate buffer (pH 7.0-9.5) and Tris-HCl buffer (pH 7.0–9.0). Selected buffer was used to perform reaction of enzyme, with different molarity ranging from 0.025 to 0.6 M. Enzyme reaction was carried out at different temperatures (30ºC to 75ºC) to work out the optimum reaction temperature. Enzyme reaction was carried out for different time periods ranging from 10 to 60 min. Starch, citrus pectin (DE-89%), apple pectin (methyl 7.8%) and amylopectin (1% each) were used as substrate to check substrate affinity of enzyme. Varied concentrations of starch were used in the range from 0.025%-0.150% (w/v). Varied concentrations of enzyme (10µl-100µl, 2.33 mg/ml protein) were used to find optimum enzyme concentration. Enzyme activity was then determined.

Results and Discussion
Isolation and screening of amylase producing bacteria
Thermophilic microorganisms are of great interest now-a-days since the enzymes produced by them are even active at high temperatures [3, 35-40]. In the present study, 23 bacterial isolates were isolated from water and soil samples, out of which 10 isolates showed zone of starch hydrolysis in starch agar plates. Among these 10 isolates, isolate K7 gave maximum production of amylase (Table 1).

Characterization of bacterial isolate
The selected isolate K7 was observed to be Gram positive and spore forming bacterium and was identified as *Bacillus licheniformis* (NCBI Accession No.: KR340466) by 16s rRNA sequencing.

Growth profile of *Bacillus licheniformis*
In the present study, the α-amylase production from *Bacillus licheniformis* was observed to increase with increase in inoculum. Its maximum activity was 0.273 U/ml at 24 h in seed medium. The organism produced maximum enzyme during early hours of growth within short fermentation time and it started declining after 24 h but the bacteria continued to grow in stationary phase which means biomass growth was independent of amylase production (Figure 1). Similarly the *Bacillus aquimaris* VITP4 gave maximum production of αamylase after 24 h of growth [41]. The maximum activity of α-amylase from *Brevibacillus borstelensis* R1 was achieved after 48 h [42]. In another study, maximum growth of the *Bacillus licheniformis* was obtained within 24 to 84 h of cultivation whereas the activity of the α-amylase reached a maximum within 36 h after inoculation [43].

Optimization Of Conditions For Maximum Production Of A-Amylase From The Selected Bacterial Isolate
Optimization of medium for the production of α-amylase from *B. licheniformis*
In the present study, out of the various media used, maximum amylase activity of *Bacillus licheniformis* (0.29 U/ml) was observed in medium M13 containing g/L (w/v) beef extract 3.0, peptone 5.0 and starch 1.0% (Table 2). However, meat extract along with peptone and starch gave maximum production of α-amylase from a bacterial isolate as reported by Haribhau *et al*. (2015) [44]. Appropriate concentration of organic and inorganic components of the production medium may enhance the organism's ability to synthesize maximum amount of α-amylase [45].

Optimization of carbon source and its concentration for the production of αamylase from *B. licheniformis*
Medium 13 supplemented with starch gave the maximum amylase production. The addition of different carbon sources affects not only the mode of amylase production but also the rate of carbohydrates metabolized [46]. However, the effect of carbon sources changes with the production strain and other conditions. Starch was observed as the best carbon source utilized by the organism [47, 48]. Similar result was also found by Goyal *et al*. (2005) for amylase production by *Bacillus licheniformis* and *Bacillus* sp. I-3 [49]. Carbon source must be added in appropriate concentration in the production medium to get maximum production of the enzyme from an organism as it represents the main energy supplement for the

growth and enzyme production. It was reported earlier that starch concentration beyond 1% in fermentation medium did not increase the amylase production [50] but the strain used in this research showed that 1.75% starch in a fermentation medium can also increase amylase production by *Bacillus licheniformis* (0.4 U/ml) while starch concentration beyond this decreased the same. In absence of any carbon source i.e. control, very low amylase production (0.12 U/ml) was observed. At low substrate concentration the active sites of enzyme are not saturated and thus the enzyme activity increased with the increase in substrate concentration [51]. Starch at a concentration of 2% gave maximum production of α-amylase from *Bacillus subtilis* and *Aspergillus awamori* respectively [52, 53].

Optimization of nitrogen source and its concentration for the production of αamylase from *B. licheniformis*

The supplementation of essential nutrients greatly affects the growth of bacteria and as nitrogen sources are required for the synthesis of amino acids and hence proteins, they may stimulate amylase production [54]. In this study, maximum amylase production was observed in the medium supplemented with peptone at a concentration 0.15%. However, minimum amylase production was observed in the medium supplemented with ammonium sulphate (0.06 U/ml). Similarly peptone was reported to be the best organic nitrogen source for high amylase production by *Streptomyces tendae* TK-VL_333 [55], *Streptomyces* sp. MSC702 [56] and *Streptomyces cheonanensis* VUK-AC [57]. However, beef extract and casein were observed to give maximum amylase production from *Brevibacillus borstelensis* R1 and *Aspergillus oryzae* respectively [42, 58]. In a previous study, 0.6% concentration of NH_4Cl gave maximum production of α-amylase from *Bacillus amyloliquefaciens* [59]. In contrast to these, 1% (w/v) of casein enzyme hydrolysate was observed to enhance α-amylase production from *Marinobacter* sp. EMB8 [60].

Effect of metal ions on the production of α-amylase from *B. licheniformis*

Metal ions are the important regulators of enzyme production [61]. In the present study it was observed that all the metal ions studied inhibited the amylase production from *Bacillus licheniformis*. Maximum inhibition was observed with Zn^{2+} (86.54%) and Hg^{2+} (84.61%) followed by Pb^{+}, Fe^{3+}, K^{+}, Cu^{2+} and Na^{+} (59.61-25.0%). However Ca^{2+} and Mg^{2+} showed minimum inhibition of 5.7 and 11.54% respectively on α-amylase production from *Bacillus licheniformis* (Figure 2). The effects of metal ions have been well studied on several amylases from fungi and bacteria. Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Mn^{2+} Mg^{2+}, Zn^{2+}, Fe^{2+} etc [62]. In a recent study, *Bacillus subtilis* gave maximum production of amylase in the presence of Mg^{2+} ions [63].

Effect of incubation temperature, pH and incubation time on the production of αamylase from *B. licheniformis*

Maximum production (0.52 U/ml) of α-amylase from *Bacillus licheniformis* was observed at 50°C of incubation temperature. Approximately 50% loss in activity was observed at 65°C of temperature (Figure 3a). These results confirm the thermophilic nature of enzyme. The organism preferred to grow in the temperature range of 40°C to 60°C. Recently similar results were observed by Abd-Elhalem *et al.* (2015) for αamylase production from *Bacillus amyloliquefaciens* [5]. However thermococcus α-amylases are optimally active at temperatures close to 80°C [64]. In contrast to this, *Penicillium fellutanum* showed an optimum production of α-amylase at a temperature of 30°C [65].

In the present study, maximum amylase production was observed at pH of 8.0 with the enzyme activity of about 0.52 U/ml (Figure 3b). All enzymes are pH sensitive; therefore pH influences stability of the enzyme [64]. Further increase in production pH beyond 8.0 led to decrease in amylase production. Similar results were observed recently by Nwokoro and Anthonia (2015) for α-amylase production from *Bacillus subtilis* CB-18 [66]. Most of the *Bacillus* strains used for α-amylase production were found to have an optimum pH between 6.0 and 7.0 for growth and enzyme production. While optimizing incubation time, maximum amylase production was found at 48 h of incubation (0.53 U/ml) (Figure 3c). Longer incubation led to accumulation of other by-products and hence depletion of nutrients [67]. Similar findings were observed on *Bacillus subtilis*, *Bacillus* sp. DLB9 [68] and *Bacillus megatarium* [69].

Optimization of inoculum age and inoculum size for the production of α-amylase from *B. licheniformis*

Maximum amylase production was shown in the medium inoculated with inoculum age of 24 h and inoculum size of 3%. It might be due to the fact that bacteria were in their active state of growth. By further increasing the age of inoculum, there was a marked decline in enzyme productivity. It might be due to the accumulation of other by-products such as secondary and tertiary metabolites or proteolysis [70]. Similarly, 24 h old inoculum was used to get maximum production of α-amylase from *Bacillus subtilis* [71], *B. amyloliquefaciens* IIB-14 [72] and *B. licheniformis* [73]. In contrast to this, *Aspergillus oryzae* gave maximum production of amylase with 48 h old inoculum [74]. Amylase production increased with the increase of inoculum size upto 3% and then it started decreasing. It might be due to the fact that at higher inoculum size led to depletion of nutrients in the media due to growth of bacteria. Lower inoculum size led to increase in fermentation time for enzyme synthesis as longer time is required to attain an optimum number of cells to utilize the substrate forming the desired product [75]. In previous studies, *Bacillus* sp. gave maximum production of amylase with an inoculum size of 2 and 2.5% [75, 76]. However in other studies 5% inoculum was optimized for α-amylase production from *Calvatia gigantea* [77] and *Thermoactinomyces sacchari* [78].

Optimization of reaction conditions for α-amylase

In the present study, α-amylase gave maximum activity with Tris-HCl (0.1 M) buffer of pH 8.0. This result indicates alkaline nature of the enzyme. The enzyme gave very low activity with buffers of acidic pH range. Similar results were observed by Dahiya and Rathi (2015) for α-amylase from *Bacillus licheniformis* [6]. However α-amylase from *Bacillus amyloliquefaciens* had maximum activity with phosphate buffer of pH 7.0 [79]. During optimization of molarity of buffer,

0.1 M Tris HCl buffer gave maximum activity which was in agreement with another study by Devi *et al*. (2012) on α-amylase from *Bacillus* sp [80]. The α-amylase gave maximum activity at 50°C of incubation temperature (Figure 4a). Further increase in incubation temperature led to denaturation of the enzyme and hence decrease in enzyme activity. Similarly at 50°C of incubation temperature, maximum activity of α-amylase from *Geobacillus* sp. NMS2 [81]. In other studies, the amylase from *Amitermes evuncifer* Silvestri [82] gave maximum activity at 50ºC. However in another study by Cordeiro *et al*. (2002), 70°C of incubation temperature was observed to be optimum for maximum activity of α-amylase from *Bacillus* sp [83]. In the present study, 10 min of incubation was observed to be optimum for maximum activity of α-amylase from *Bacillus licheniformis*. Prolonged incubation probably denatures the enzyme. In other studies, only 5 min of incubation was observed to give optimum activity of α-amylase from *Bacillus licheniformis* ATCC 6346 and *Bacillus subtilis* KIBGE HAS respectively [84, 85]. Amylase from *Rhizopus oryzae* showed incubation time of 40 min for maximum activity of enzyme [86].

The α-amylase from *B. licheniformis* showed highest affinity towards starch as substrate at a concentration of 0.15% (w/v). Similarly starch was observed to be the best substrate to get maximum activity of amylase from *Bacillus amyloliquefaciens* [79]. However amylase from *Filobasidium capsuligenum* showed higher affinity towards amylase as substrate [87]. In general, increase in substrate concentration increases the speed of the reaction as active sites of the enzyme bind to the substrate. Once all the active sites got bound to the substrate, further increase in substrate concentration will have no effect on activity. However, 1% (w/v) concentration of soluble starch was observed to be best for maximum activity of α-amylase from *Bacillus subtilis* [84]. Maximum enzyme activity (1.49 U/ml) was found with 30 µl of enzyme (0.07 mg) as shown in Figure 4b. On further increase in concentration, the enzyme activity decreased.

Conclusion

Thermophilic α-amylase producing *Bacillus licheniformis* was isolated from hot spring of Himachal Pradesh and almost 5-fold increase in activity of α-amylase was observed after complete optimization. The thermophilic and alkaliphilic property of the enzyme suggest its potential application in starch, detergent and textile industries.

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Table 1: Activity of α-amylase from different isolates

Isolate Number	Enzyme activity (U/ml)
K1	0.023
K2	0.021
K3	0.020
K4	0.021
K5	0.018
K6	0.020
K7	**0.29**
K8	0.19

Table 2: Effect of different media on production of amylase from *B. licheniformis*

Medium	Enzyme activity (U/ml)	Standard Deviation
M1	0.18	0.004
M2	0.03	0.009
M3	0.09	0.001
M4	0.09	0.002
M5	0.06	0.006
M6	0.24	0.01
M7	0.21	0.003
M8	0.16	0.004
M9	0.03	0.002
M10	0.05	0.001
M11	0.02	0.007
M12	0.12	0.003
M13	0.29	0.004
M14	0.15	0.003
M15	0.23	0.007
M16	0.16	0.009
M17	0.19	0.002

Figure 1: Growth profile of *Bacillus licheniformis*

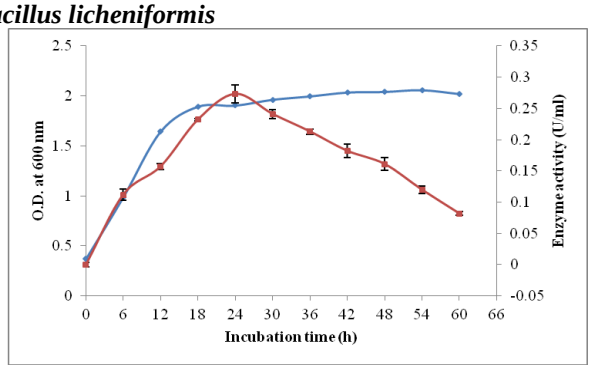

Figure 2: Effect of metal ions on production of amylase from *B. licheniformis*

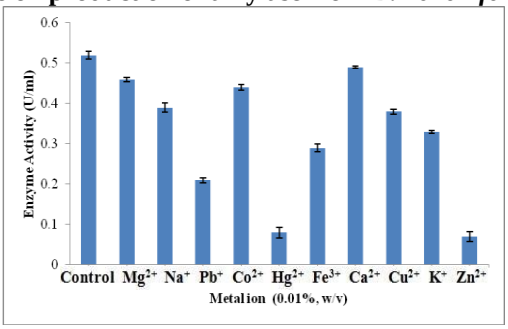

Figure 3: a. Effect of incubation time b. incubation temperature and c. pH on production of α-amylase from *Bacillus licheniformis*

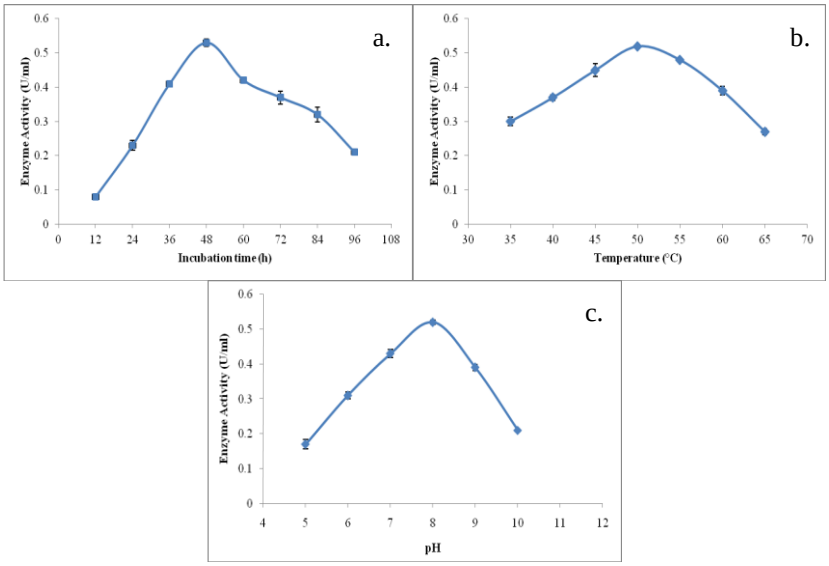

Figure 4: a. Effect of incubation temperature and b. enzyme concentration on activity of αamylase from *Bacillus licheniformis*