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High Level Production of Extracellular α-Amylase from *Bacillus licheniformis* ATCC 12759 in Submerged Fermentation

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Abstract

Alpha amylase (E.C. 3.2.1.1) of Bacillus licheniformis ATCC 12759 was shown to produce extracellular α-amylase in submerged fermentation.Various nutrients belonging to three categories, carbon, nitrogen and amino acid sources, were investigated in terms of their effect on the production of extracellular α-amylase by Bacillus licheniformis ATCC 12759. Amongst carbon sources sugars, arabinose, sucrose, corn flour and corn starch supported maximum amylase production. Casein, sodium nitrate, ammonium sulphate, ammonium chloride, and ammonium nitrate where the best organic sources and inorganic sources respectively. Among the amino acid sources tested, L-cysteine, L-tryptophane, L-valine, L-phenylalanine, L-methionine and L-lysine were favored the production respectively. $FeSO_4$, $ZnSO_4$ and $CuSO_4$ inhibited α-amylase production. Maximum α-amylase production (1074.8±35.0 U/mg) was obtained in a medium containing 0.01% L-cysteine in 72 h at 37 ^oC.

Keywords: *Bacillus licheniformis* ATCC 12759, α-amylase, submerged fermentation, enzyme production

Introduction

α-Amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of internal α-1,4-*O*- -glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products [1]. These enzymes account for about 30 % of the world's enzyme production [2]. Amylases are among the most important enzymes and are of great significance for biotechnology. α-Amylases have potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries. However, with the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medicinal and analytical chemistry, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries [3-6].

α-Amylase has been derived from several fungi, yeasts and bacteria. The amylases of microorganisms have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal α- amylases [7]. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry [6]. Members of the genus *Bacillus* produce a large variety of extracellular enzymes of which amylases are of particularly significant industrial importance [8].

Submerged fermentation has been defined as fermentation in the presence of excess water. Almost all the large-scale enzyme producing facilities are using the proven technology of SmF due to better monitoring and ease of handling [9]. To meet the growing demands in the industry it is necessary to improve the performance of the system and thus increase the

yield without increasing the cost of production [10]. The optimization of fermentation conditions, particularly physical and chemical parameters, are important in the development of fermentation processes due to their impact on the economy and practicability of the process [11]. The growth and enzyme production of the organism are strongly influenced by medium composition thus optimization of media components and cultural parameters is the primary task in a biological process [12].

Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques [13]. Selection of appropriate carbon and nitrogen sources or other nutrients is one of the most critical stages in the development of an efficient and economic process [14]. According to the experimental data presented in this study, the *Bacillus licheniformis* ATCC 12759 used in this work could be used for the production of α-amylase, the most commercially interesting enzymes, in one fermentation process using inexpensive nutrients.

Materials and methods

Microorganism

α-Amlyase producing *Bacillus licheniformis* ATCC 12759 which was procured from MicroBioLogics, Inc. was used as biological material. *B. licheniformis* ATCC 12759 was grown on nutrient agar at 37°C for 24 h for inoculum preparation. A loopful of the growth was transferred to Laura broth (LB) liquid medium (1% yeast extract, 0.5% peptone, 0.5% NaCI, (w/v), pH 7.0).

Enzyme production

The organism was grown at 37°C for 5 days in 25 ml of medium with shaking on a shaker (150 rpm). Samples were taken from 12 to 120 h. The supernatant of the culture after centrifugation (10.000 rpm, 10 min) at 4°C was used to determine extracellular amylase activity.

Enzyme assay

α-Amylase activity was determined by the procedure of Bernfeld using soluble starch as a substrate [15]. The reaction mixture containing 200 µl of 1% substrate (w/v) in 0.1 M phosphate buffer (pH: 7.0) and 150 µl of enzyme solution was incubated for 30 min at 37°C. The reaction was stopped by adding 400 µl of 3,5-dinitrosalicylic acid solution followed by heating in a boiling water bath for 5 min and cooling at room temperature and then 8 ml of deionized water was added. Absorbance of each solution containing the brown reduction product was measured at 489 nm in a UV-visible spectrophotometer.

One unit (U) of α-amylase activity was defined as the amount of enzyme that releases 1 µ mol of reducing sugar as maltose per minute, under assay conditions.

All experiments were conducted in triplicate and the mean at three with standard deviation (SD) was represented.

Assay of protein concentration

The protein concentration was determined by the Lowry's method using bovine serum albumin used as Standard [16].

Effect of incubation period

The effect of incubation period was determined by incubating production medium for different incubation periods (12, 24, 48, 72, 96, and 120 h) at 37ºC taking other conditions into consideration.

Effect of carbon sources

Different carbon sources such as soluble starch, wheat starch, potato starch, corn starch, wheat flour, rice flour, corn flour, glycerol, mannose, xylose, lactose, galactose, arabinose, glucose, sucrose, and fructose were employed to find the suitable carbon source for α-amylase production by *B. licheniformis* ATCC 12759. All these sources were studied at 1% (by mass per volume) initial concentrations.

Effect of nitrogen sources

Two categories, viz. organic nitrogen sources and inorganic nitrogen sources were employed. The growth medium was initially supplemented with different organic nitrogen sources, i.e. yeast extract, tryptone, beef extract, peptone, casein, urea, soy flour each at 1% (by mass per volume). Among the inorganic nitrogen sources, ammonium nitrate (NH_4NO_3) ammonium chloride (NH_4CI), ammonium sulphate ($(NH_4)_2SO_4$), and sodium nitrate ($NaNO_3$) again at 1% (by mass per volume) were tested.

Effect of amino acid sources

To study the effect of amino acid sources on production of α-amylase glycin, L-lysine, L-tyrosine, L-cysteine, glutamic acid, L-alanine, L-phenylalanine, L-isoleusine, L-valine and L-methionine selected as amino acid source. All these sources were studied at 0.01% (by mass per volume) initial concentrations.

Effect of metal salts

The effect of metal salts on α-amylase production is determined by adding different metal salts in the fermentation medium. The metal salts selected for present study are $FeSO_4$, $MgSO_4$, $CaCl_2$, $CuSO_4$, and $ZnSO_4$, at 0.1% concentration.

Results and Discussion

Microorganisms like fungi and bacteria have been extensively screened for α-amylase production [17]. In the bacteria, *Bacillus* species such as *B. subtilis, B. licheniformis* and *B. stearothermophilus* can be used for the better production of α-amylase in shake flask [18,19].

The incubation time is governed by characteristics of the culture and also based on growth rate and enzyme production. At different time courses the production of amylase is shown in Fig 1. Maximum amylase production was obtained at 72 hours of incubation. A prolonged incubation time beyond this period did not increase the enzyme yield. Enzyme production is related to growth of the microorganism. Growth of the organisms would have reached a stage (due to insufficient nutrients) that indirectly stimulates production of secondary metabolites [20]. A similar result was reported by Nusrat and Rahman [21], Akcan et al. [22] and Božić et al. [23].

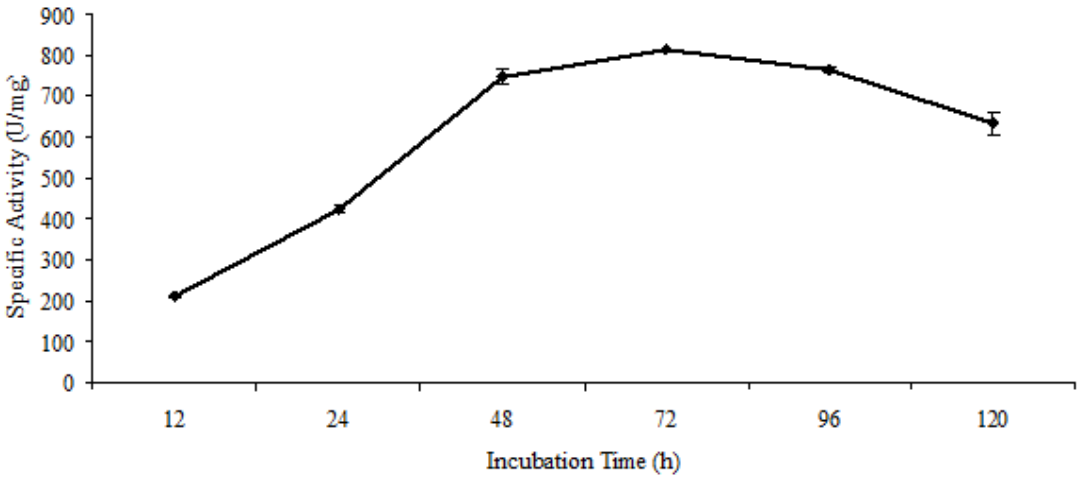

Figure 1. Effect of incubation time on the production of *Bacillus licheniformis* ATCC 12759 α-amylase.

The nature and amount of carbon source in culture media is important for the growth and production of extracellular amylase in bacteria. Arabinose, sucrose, corn flour and corn starch enhanced the amylase activity while other carbon sources and, specially, glycerol, wheat starch, wheat flour and rice flour repressed amylase production (Table 1). These results suggest that amylase production in *B. licheniformis* ATCC 12759 is induced by some carbon sources. It has been reported that the synthesis of carbohydrate degrading enzymes in most species of the genus *Bacillus* is subjected to catabolic repression by readily metabolisable substrates such as glucose and fructose [24]. Therefore, our results are in good agreement with the findings of these studies. Media containing starch rich flours such as corn were suitable for the production of α-amylase. It is emphatically known that higher yields of amylase can be obtained in media with complex raw materials containing starch from maize, barley, wheat and malt [25]. The superiority of amylase production with complex substrates has been earlier reported [26]. Natural sources could serve as economical and readily available raw material for production of valuable enzymes. Hence, these starch-rich flours may prove useful as cheaper alternative sources of carbon and energy for the bacterial production of amylases [27].

Table 1. Effect of carbon sources on the production of *Bacillus licheniformis* ATCC 12759 α-amylase.

Carbon source 1% (w/v)	Specific Activity(U/mg)
Control	787.3±25.3
Soluble starch	666.4±22.8
Wheat starch	70.9±10.7
Potato starch	397.2±12.3
Corn starch	794.2±47.2
Wheat flour	69.5±21.3
Rice flour	71.7±13.6
Corn flour	901.4±26.2
Glycerol	50.7±9.8
Mannose	585.8±72.5
Xylose	207.2±28.6
Lactose	473.2±62.0
Galactose	523.4±17.4
Arabinose	937.0±50.7
Glucose	698.0±12.4
Sucrose	844.1±78.3
Fructose	722.8±49.9

The nature of nitrogen source is important information of amylase production. The influence of organic and inorganic nitrogen sources on amylase production was determined (Fig.2). Among the different organic nitrogen sources tested, casein (922.4±44.2 U/mg) was found to be a good nitrogen source for α-amylase production. Similarly we reported casein was found to be a good nitrogen source for α-amylase production from *B. subtilis* RSKK96 [23]. Various other organic nitrogen sources have also been reported to support maximum α-amylase production by various *Bacillus* species [8, 14, 22, 23, 25, 28]. Other nitrogen containing compounds such as organic did not support α-amylase production. It was clear though that certain organic compounds may be necessary for the biosynthesis of α-amylase. All the different inorganic nitrogen sources tested α-amylase synthesis. Aiyer [25] reported ammonium hydrogen phosphate to be a better nitrogen source for enzyme production by *B. licheniformis* SPT 278 than other tested inorganic nitrogen sources. Similarly, Kundu et al. [29] showed that sodium nitrate and ammonium nitrate were the best nitrogen sources for maximum amylase production. Coleman and Elliott [30] stated that ammonium salts were stimulators of *B. subtilis* amylase production. Hence, our findings are in a good agreement with the findings of these studies.

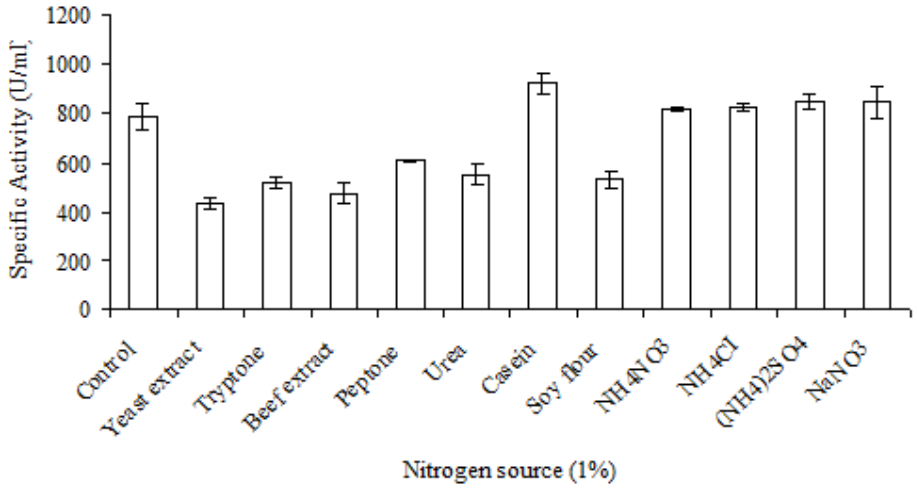

Figure 2. Effect of nitrogen sources on the production of *Bacillus licheniformis* ATCC 12759 α-amylase.

The effect of amino acids supplementation on enzyme production was also studied. L-cysteine, L-tryptophane, L-valine, L-phenylalanine, L-methionine and L-lysine were found to be the ideal amino acids sources, respectively (Fig 3). Supplementation of the L-cysteine (1074.8±35.0 U/mg) in fermentation medium was enhanced α-amylase production 136%. Rasooli et al. [8] reported that tryptophane was found to enhance the enzyme productivity to 202% as compared to the basal medium whereas peptone and lysine at 0.5% level showed a strong repression on α-amylase production from *Bacillus* spp. However, the role of amino compounds was considered to be neither as nitrogen nor as a carbon source, but as stimulators of amylase synthesis and excretion [31]. It has been reported that only asparagine gave good enzyme yields while the importance of arginine for α-amylase production from *B. subtilis* has also been well documented [29, 32]. The amylase synthesis by several microorganisms has been correlated to the presence or absence of different nitrogen sources and various amino acids in

the growth medium. The differences in nutritional requirements of various α-amylase producing organisms or microbial strains could be attributed to the difference in their genetics [8].

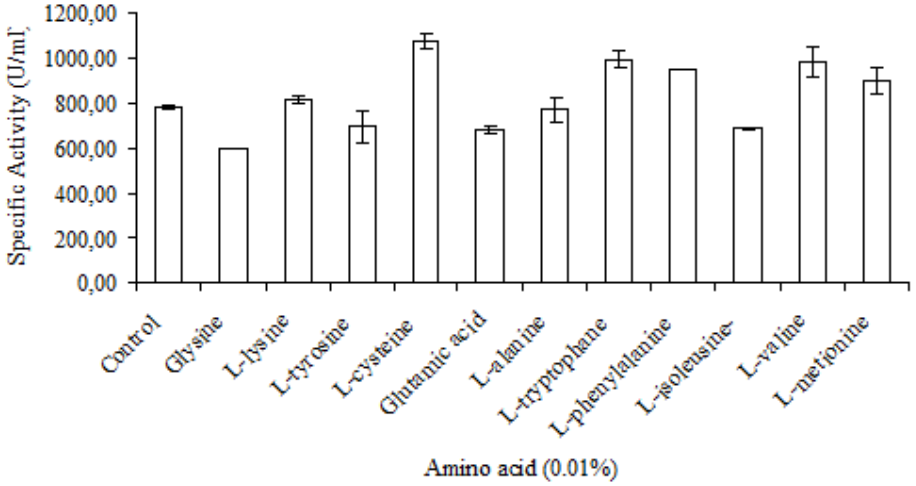

Figure 3. Effect of amino acid sources on the production of *Bacillus licheniformis* ATCC 12759 α-amylase.

Supplementation of salts of certain metal ions provided good growth of microorganisms and thereby better enzyme production (as most α-amylases are known to be metalloenzymes) [13]. α-Amylase production by *B. licheniformis* ATCC 12759 was increased in the presence of 0.1% $CaCl_2$ (854.6±41.8 U/mg) similar to *Bacillus* sp. TSCVKK and *Bacillus* sp. 64 [33, 34]. Ca^{+2} had significant effects on the metabolism and physiology of bacteria, and there was also found to be an effect on enzyme activity and stabilization in the defense against proteases [35]. The other metal salts $MgSO_4$ tested decreased enzyme production (Fig 4). The inhibitory effects of some of the salts may be related to the pH changes associated with their use in the medium. 0.1%, $FeSO_4$, $CuSO_4$, and $ZnSO_4$ completely inhibited α-amylase production. The results are also confirmed by Kıran et al. [33] who stated that the presence of $ZnSO_4$ had a potent inhibitory effect on the production α-amylase from *Bacillus* sp. K-12.

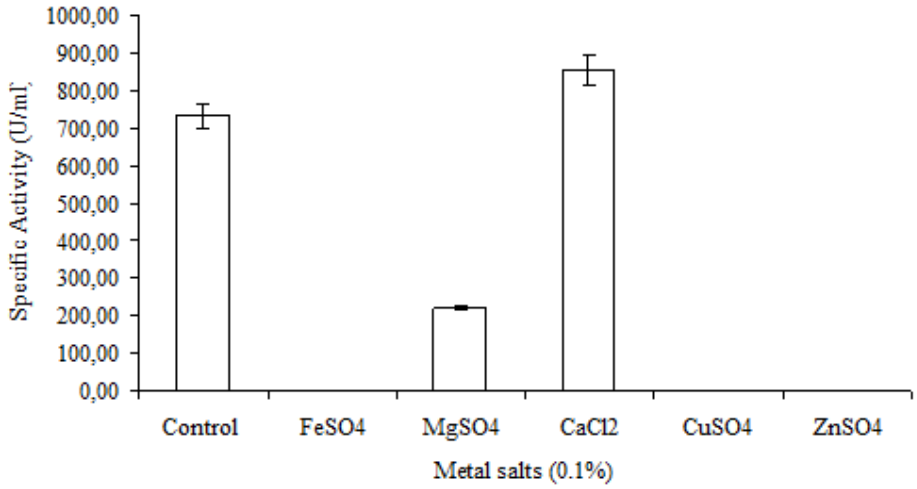

Figure 4. Effect of metal salts sources on the production of *Bacillus licheniformis* ATCC 12759 α-amylase

The results obtained in this study show that there is appreciable high production. This suggests that *B. lichenisformis* ATCC 12759 can be a potential producer of extracellular α-amylase which could find applications in industry and biotechnology. The enzyme thus produced is presently under optimization. Due to the importance of these findings, further studies will be carried on in order to commercialize the production process after necessary optimization for enhanced enzyme production.

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