

Solid State Fermentation for Cultural Conditions Optimization and Production of α -Amylase from *Bacillus licheniformis* ATCC 12759

Bacillus licheniformis ATCC 12759'dan α -Amilazın Üretimi ve Kültür Koşullarının Optimizasyonu için Katı Faz Fermentasyonu

Research Article

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ABSTRACT

The aim of this paper is to study influence of the certain production parameters of α -amylase by *Bacillus licheniformis* ATCC 12759. Various agroresidues as substrate were studied for enzyme production. The highest enzyme production was expressed with rice bran as units per mass of dry substrate (1399.8 \pm 6.6 U/mg). Optimization parameters of α -amylase production were carried out with solid state fermentation (SSF). Solid waste from rice bran used as the basic nutrient source. Supplementation with carbon and metal salt sources decreased the enzyme production. Certain fermentation parameters involving incubation time, incubation temperature, inoculum level, moisture level, extraction medium, initial pH and medium volume were studied separately. Maximal amount of α -amylase production (2094.9 \pm 53.1 U/mg) was obtained inoculum level 30%, moisture level 20%, initial pH 6.5 at 37°C for 48 h with supplementation of ammonium chloride.

Key Words

Bacillus licheniformis ATCC 12759, rice bran, α -amylase, solid state fermentation, optimization, production.

ÖZ

Bu çalışmanın amacı *Bacillus licheniformis* ATCC 12759'dan α -amilazın belirli üretim parametrelerinin etkisini incelemektir. Enzim üretimi için substrat olarak çeşitli tarımsal atıklar çalışılmıştır. En yüksek enzim üretimi kütle başına (1399.8 \pm 6.6 U/mg) pirinç kabuğu ile ifade edildi. α -Amilaz üretiminin optimizasyon parametreleri katı faz fermantasyonu (KSF) ile gerçekleştirildi. Katı atık olarak pirinç kabuğu temel besin kaynağı olarak kullanıldı. İnkübasyon zamanı, inkübasyon sıcaklığı, inokulum oranı, nem oranı, özütleme ortamı, başlangıç pH'sı ve ortam hacmini içeren belirli fermantasyon parametreleri ayrı ayrı çalışıldı. α -Amilaz üretiminin en yüksek miktarı (2094.9 \pm 53.1 U/mg) 48. saatte 37°C'de başlangıç pH 6.5 %20 nem oranı ve %30 aşılama oranı ile amonyum klorürün eklenmesi ile elde edilmiştir.

Anahtar Kelimeler

Bacillus licheniformis ATCC 12759, pirinç kabuğu, α -amilaz, katı faz fermantasyonu, optimizasyon, üretim.

Article History: Received: Apr 07, 2017; Revised: May 21, 2017; Accepted: Sep 12, 2017; Available Online: Dec 25, 2017.

DOI: 10.15671/HJBC.2018.202

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INTRODUCTION

Amylase (α -1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) is an enzyme that degrades starch, first to oligosaccharides and then in turn to maltose and glucose, by hydrolyzing α -1,4-glucan bonds [1]. Amylases are employed in industries for different purposes; glucose and maltose-forming α -amylases in alcohol fermentation and sugar syrup formulation, and malto-oligosaccharide-forming α -amylases in food processing [2,3]. Amylases also play a significant role in starch, detergent, beverage and textile industries and its commercial production from microorganisms represent 25-33% of the world enzyme market [4,5]. α -Amylases are ubiquitous enzymes produced by plants, animals and microbes. In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization [6,7].

Commercial α -amylase is usually produced by SmF but the cost of enzyme production in submerged fermentation (SmF) is high. The contents of synthetic media are very expensive and these contents might be replaced with more economically available agricultural by-products for the reduction of cost of the medium [8]. The use of agricultural wastes makes solid-state fermentation (SSF) an attractive alternative method [9,10]. SSF is defined as the cultivation of microorganisms on moist solid support, either on inert carriers or insoluble substrates that can, in addition be used as carbon and energy source. The aim of SSF is to bring the cultivated microorganisms into tight contact with the insoluble substrate and thus to achieve the highest substrate concentration during fermentation [11]. Bacterial systems are now being increasingly investigated for the production of enzymes and metabolites by solid-state fermentation [12].

SSF has numerous advantages, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, and requirement for simple fermentation equipments than SmF [1,13,14]. The major factors that affect microbial synthesis of enzymes in a SSF system include selection of a suitable substrate and microorganism,

particle size of the substrate, inoculum concentration and moisture level of the substrate [10,15].

Development of an economical production medium requires the selection of a carbon, nitrogen, phosphorus, sulfur, potassium and trace element source as well as energy source that will support not only good microbial growth but also maximize product yield, reduce synthesis of compounds closely related to the product and enhance product recovery [16]. The objective of the present study was to screen a variety of agro-industrial waste residues as substrates for α -amylase production by *Bacillus licheniformis* ATCC 12759 in SSF and optimization of fermentation conditions with respect to moisture content and extraction medium of the best fermentable material, incubation time, incubation temperature, initial pH, volume of fermentation medium, supplementation of carbon, nitrogen and metal salt sources for α -amylase production in SSF.

MATERIALS and METHODS

Chemicals

All reagents used in this study were purchased from Sigma (USA).

Microorganism and Growth Medium

α -Amylase producing *B. licheniformis* ATCC 12759 which was purchased 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 Luria broth (LB) liquid medium (1% yeast extract, 0.5% peptone, 0.5% NaCl, (w/v), pH 7.0).

Substrate

Wheat bran (WB), rice bran (RB), cotton stalk (CS), crushed maize (CM), millet cereal (MC) were obtained in Diyarbakır, Turkey. The substrates were ground into coarse powder with a blender and used. The rice bran which found as best substrate was used to be solid substrate. This is cheap and readily available in the local market, Diyarbakır, Turkey.

Solid State Fermentation

In an attempt to choose a potential substrate for SSF which supports α -amylase production, vario-

us agro residues like wheat bran (WB), rice bran (RB), cotton stalk (CS), crushed maize (CM), millet cereal (MC) were screened individually. SSF was carried out by taking 3 g of dry substrate in a 100 mL Erlenmeyer flask to which distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121°C for 15 min. Flasks with inoculated with 3.0 ml of spore suspension (2.10^7 CFU/mL) were shaken at 150 rpm at 37°C for 144 h. The contents of the flasks were harvested and assayed every 24 h.

Enzyme Extraction

The enzyme from the fermented bacterial bran was extracted twice with tap water. The slurry was squeezed through damp cheesecloth. Extracts were pooled and centrifuged at 4°C for 15 min at 10.000 rpm to separate small particles of different substrates, cells and spores. The yellow, clear supernatant was used in enzyme assays.

Enzyme Assay

α -Amylase activity was determined by the procedure of Bernfeld using soluble starch as a substrate [17]. The reaction mixture containing 200 μ l of 1% substrate (w/v) in 0.1 M phosphate buffer (pH 7.0) and 150 μ l of enzyme solution was incubated for 30 min at 37°C. The reaction was stopped by adding 400 μ l 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 and expressed as U/mg of dry substrate.

All the experiments are independent of each other. Results are represented as mean \pm S.D. of at least three experiments.

Protein Determination

The protein amount was determined by Lowry method by using bovine serum albumine (BSA) as standart [18].

Effect of Process Parameters on α -Amylase Production in SSF

Various process parameters influencing enzyme production during SSF were optimized. The strategy was to optimize each parameter independently of the others and subsequently optimal conditions were employed in all experiments. The rice bran was employed for further optimization of process parameters, namely incubation time (24-96 h), incubation temperature (30, 37, 40, 45 and 50 °C), inoculum size (10-80%), moisture level (20, 30, 40, 50, 60 and 80%), extraction medium (distilled water, tap water, 50 mM NaCl, 0.1 M phosphate buffer (pH 7.0), 1% solution of various detergents like Tween 40, CHAPS (3-[(3-Cholamido-propyl)-dimethylammonio]-propane- sulfonate), sodium dodesil sulfate (SDS) and Triton X-100), initial pH of the medium (pH 5.0-9.0) and volume of fermentation medium (100, 250, 500 and 1000 ml erlenmeyer flaks) were optimized. To study the efficacy of various inducers, the medium was supplemented independently with 1% mannose, xylose, lactose, sucrose, fructose, galactose, glucose, and arabinose. While nutrient supplementation such as inorganic nitrogen sources 1% (by mass) (ammonium nitrate, sodium nitrate, ammonium chloride and ammonium sulphate), organic nitrogen sources (peptone, tryptone, yeast extract, beef extract, urea, and casein), and added metal salts 0.1% (by mass) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and CaCl_2 were optimised. For each experimental variable all other parameters were kept at their optimal level.

RESULTS and DISCUSSION

It has been well documented that *Bacillus* species served as the most important sources of α -amylase[5,8,10,11]. In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation [19]. In this study in order to reduce the cost of α -amylase production, agro-industrial residues or cheap materials were chosen as culture media. As it is shown in Table 1, A high titer of α -amylase production (1399.8 ± 6.6 U/mg) was obtained in a medium containing rice bran (RB) alone as the substrate.

Table 1. Effect of different agro-industrial wastes on *B. licheniformis* ATCC 12759 α -amylase production by solid state fermentation.

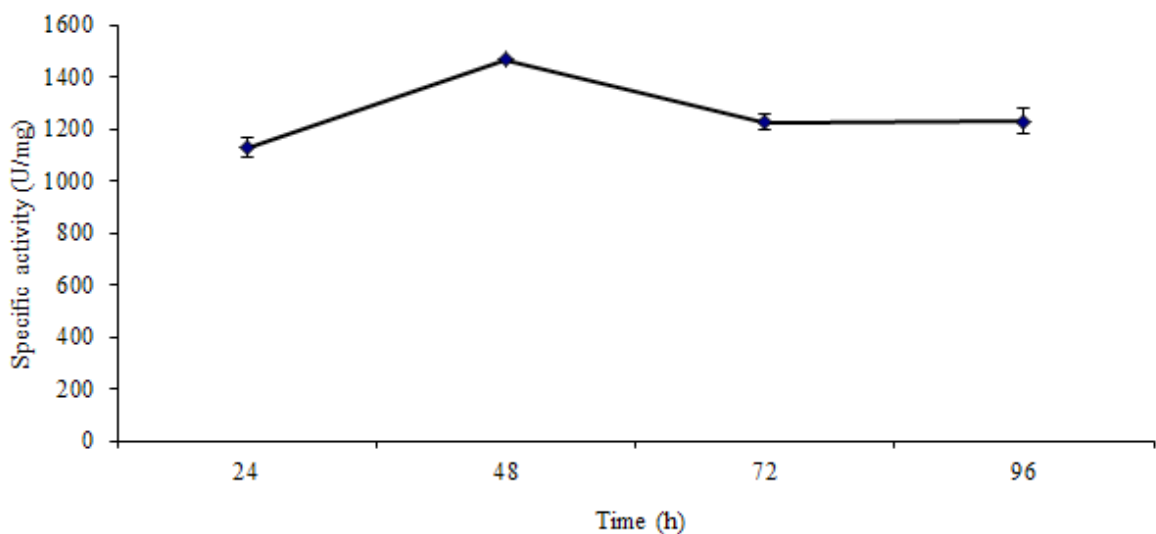
Hour	Substrates				
	Cotton stalk (CS)	Rice bran (RB)	Millet cereal (MC)	Wheat bran (WB)	Crusded maize (CM)
	Enzyme Production (U/mg)				
24	448.7±30.2	1127.8±15.0	781,3±72.3	639.8±7.5	428.2±34.2
48	519.4±5.8	1399.8±6.6	786.6±3.1	202.9±1.6	382.0±18.1
72	527.5±3.3	1225.6±36.6	455.4±15.9	196.7±4.7	282.8±21.3
96	1052.4±42.1	1121.7±23.8	143.1±18.3	203.9±3.8	232.0±15.8

Recently, SSF of rice bran was developed to replace wheat bran in the tropics [20]. Wheat bran is reported to give higher enzyme yield compared to rice bran and other agro-wastes [9], while comparable α -amylase production has been reported using rice components versus wheat bran as solid substrate for fermentation [13,21-23]. However, using this substrate also solves pollution problems. Due to the superior potential of RB in inducing the α -amylase production by *B. licheniformis* ATCC 12759 in SSF, this substrate was selected for subsequent experiments (optimization of process parameters)

The incubation time was governed by the characteristics of the culture, based on the growth rate and the enzyme production pattern [24]. Maximum enzyme production (1466.7±2.1 U/mg) was observed after 48 h, which decreased with further incubation (Figure 1). The reason for this might have

been due to the denaturation of the enzyme caused by the interaction with other components in the medium [25].

Temperature is one of the important factors, which strongly affect the SSF process. It plays a significant role in development of the biological process as it influences protein denaturation, enzyme inhibition and cell growth [26]. It has been reported that during microbial cultivation in SSF, the temperature of the fermenting bed increased, which exerted harmful effects on the microbial activity [27,28]. Investigation of the effect of temperature on enzyme production during fermentation showed that the optimum temperature for maximum yield of α -amylase was 37°C for rice bran (Figure 2). Maximum production at lower temperatures may be advantageous as it can reduce the rate of evaporation during incubation [22]. Previously similar results were reported for

**Figure 1.** Effect of incubation time on the production of α -amylase by *B. licheniformis* ATCC 12759. Inoculum size 30% (by volume per mass), Moisture level 20%, Extraction medium with tap water, Initial pH 6.5, Incubation temperature 37°C.

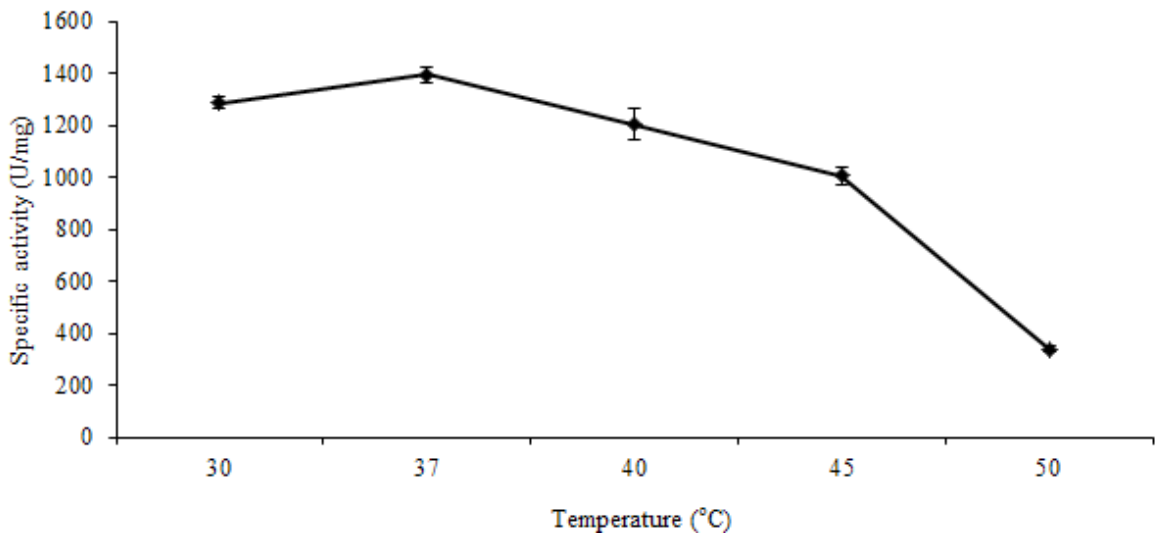


Figure 2. Effect of incubation temperature on the production of α -amylase by *B. licheniformis* ATCC 12759. Inoculum size 30% (by volume per mass), Moisture content 20%, Extraction medium with tap water, Initial pH 6.5, Incubation time 48.

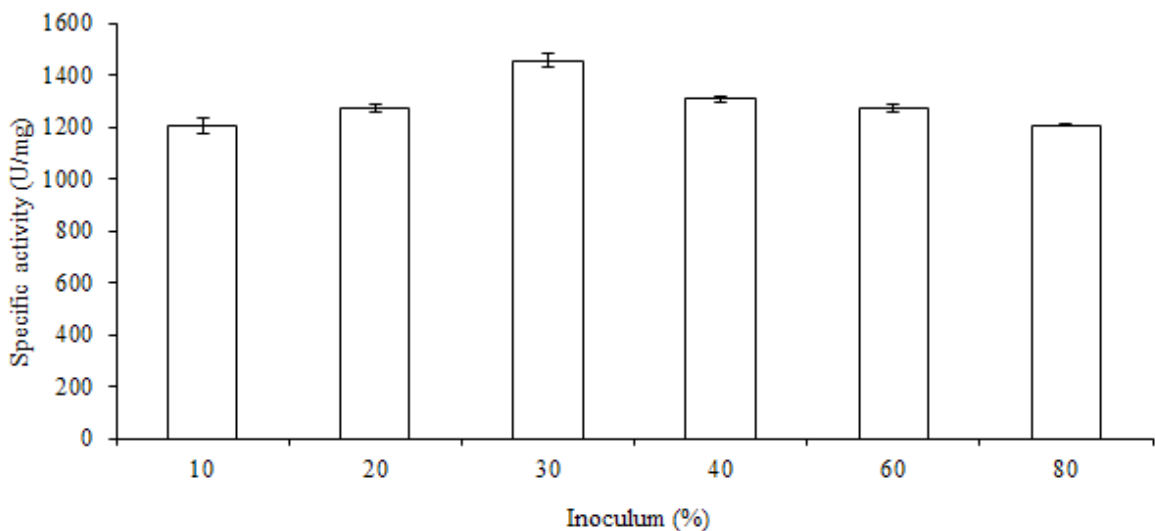


Figure 3. Effect of inoculum size on the production of α -amylase by *B. licheniformis* ATCC 12759. Moisture level 20%, Initial pH 6.5, Extraction medium with tap water, Incubation temperature 37°C, Incubation time 48.

production of α -amylase [10,23,29,30]. It has also been reported that the metabolic heat generated during microbial cultivation in SSF exerts harmful effects on the microbial activity and thus the initial set temperature is vital [31].

There is a great influence of inoculum's concentration in enzyme production and therefore, this parameter should be given a proper consideration for optimum level of α -amylase production [32]. Various inoculum levels were tried to determine their effect on enzyme production. The higher enzyme production (1453.1 ± 36.3 U/mg for rice bran) was

obtained at 30% inoculum level, as compared to lower or higher inoculum levels (Figure 3). The results from this study indicate that 30% inoculum size was optimal, balancing enzyme and biomass production.

Moisture content of the substrate is one of the critical factors influencing the outcome of SSF, and is governed by the water-holding-capacity of the substrate, the type of end-product, and the requirement of the microorganism. The inter particle mass transfer within the solid phase to the growing microorganism depends on the substrate characteristics and the moisture content [33] The moisture content

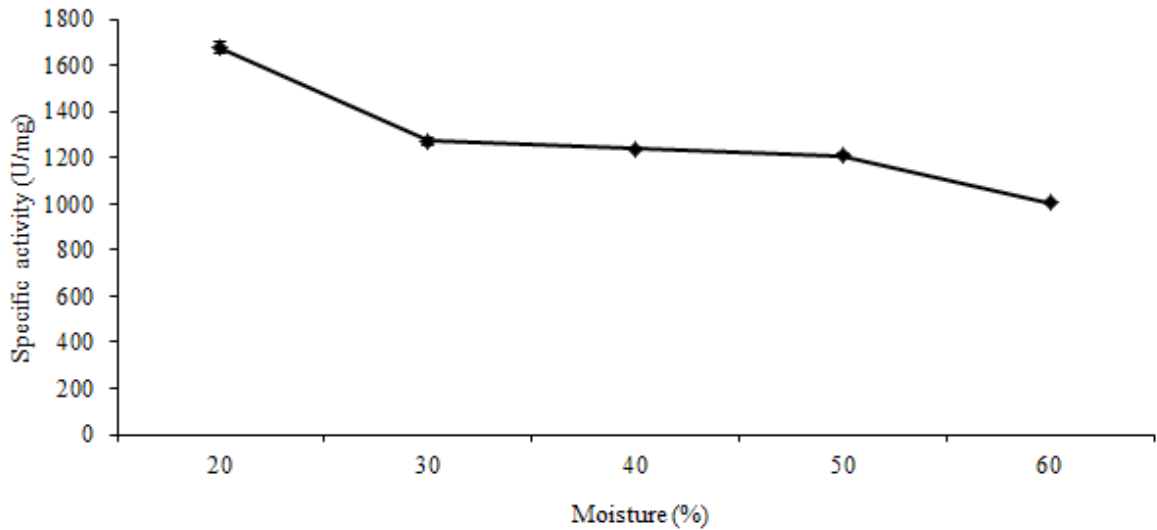


Figure 4. Effect of moisture level on the production of α -amylase by *B. licheniformis* ATCC 12759. Inoculum size 30% (by volume per mass), Extraction medium with tap water, Initial pH 6.5, Incubation temperature 37°C, Incubation time 48.

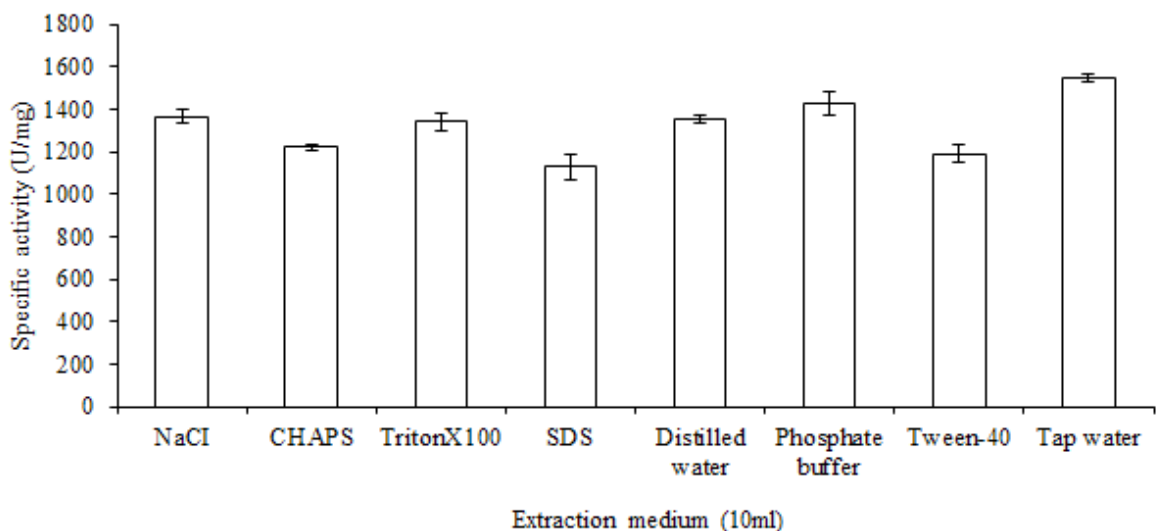


Figure 5. Effect of extraction medium on the production of α -amylase by *B. licheniformis* ATCC 12759. Inoculum size 30% (by volume per mass), Moisture content 20%, Initial pH 6.5, Incubation temperature 37°C, Incubation time 48.

of the medium changes during fermentation as a result of evaporation and metabolic activities and thus optimum moisture level of the substrate is therefore most important [13]. In the present study, high enzyme production was obtained when the moisture level was maintained as 20% in comparison with that at other moisture levels (30,40,50 and 60%) (Figure 4). Increase in moisture level is believed to reduce the porosity of the solid substrate, thus limiting oxygen transfer [25].

To obtain the enzyme extraction from fermented biomass is important. The medium

used for the extraction of crude enzyme from the fermented matter was found to have a profound effect on the enzyme yield [31]. The effect of different solvents on enzyme extraction is presented in Figure 5. Maximum enzyme production (1547.3 \pm 17.0 U/mg) was obtained with tap water, which supported amylase production.

pH is among the other most important factors for any fermentation process and is dependent upon the type of the moistening agent used in the medium. Each microorganism possesses a pH range for its growth and activity with a optimum

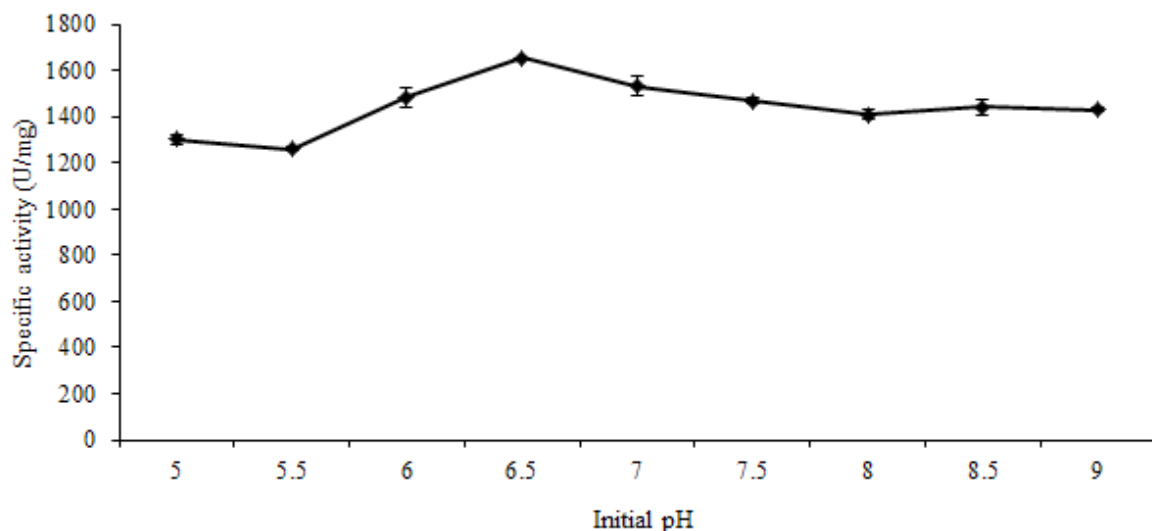


Figure 6. Effect of initial pH on the production of α -amylase by *B. licheniformis* ATCC 12759. Inoculum size 30% (by volume per mass), Moisture content %20, Extraction medium with tap water, Incubation temperature 37°C, Incubation time 48.

value in between the range [23]. The pH change observed during the growth of the organism also affects product stability in the medium. Most of the *Bacillus* strains used commercially for the production of bacterial α -amylases by SmF have an optimum pH between 6.0 and 7.0 for growth and enzyme production. This is also true of strains used in the production of the enzyme by SSF [24]. In the present study, the initial pH of the fermentation medium was founded to 6.5 (Figure 6). An attempt to overcome the problem of pH variability during the SSF process is obtained by the substrate formulation considering the buffering capacity of the different components employed or by the use of buffer formulation with components that have no deleterious influence on the biological activity [23,25]. Generally, agro industrial wastes possess unique buffering action and have advantages for enzyme production. The subsequent experiments, the initial pH of the fermentation medium was adjusted to 6.5.

Most of the laboratory studies on the production of enzymes using the SSF technique have employed Erlenmeyer flasks and trays [22-24,34-36]. To improve for a large-scale SSF, α -amylase production was investigated in four different fermentation medium sizes (100, 250, 500 and 1000 mL erlenmeyer flasks). When solid state fermentation with *B. licheniformis* ATCC 12759 was carried out in erlenmeyer flasks of various sizes with corresponding increase in with

different quantities of rice bran moistened with appropriate amounts of tap water, maximum enzyme production (1642.6 ± 52.7 U/mg) observed in 500 ml erlenmeyer flasks containing 10 g of rice bran comparison with control (containing 2 g of rice bran) (Figure 7). This result suggested that the possibility of large scale production of *Bacillus licheniformis* ATCC 12759 under SSF.

The supplementation of rice bran with the different carbon sources; mannose, xylose, lactose, sucrose, fructose, galactose, glucose, and arabinose at 1% (w/v) concentration on the production of α -amylase by *B. licheniformis* ATCC 12759 was investigated in order to obtain a suitable medium for industrial enzyme production. In our studies, as shown in Table 2 in comparison with the control (1800.6 ± 18.2 U/mg), there was no significant increase in enzyme yield in the case of the supplementation of carbon sources. All carbon sources resulted in suppression of α -amylase production by *B. licheniformis* ATCC 12759 which might be due to catabolic repression of α -amylase synthesis [37]. In a sharp contrast to this observation, many of the authors have reported either absence or a minor catabolic repression of α -amylase synthesis by microbes in SSF in presence of glucose [23,28]. Therefore, it might be reasonable to assume that the effect of glucose on induction of α -amylase synthesis is dependent on the microbial species [32].

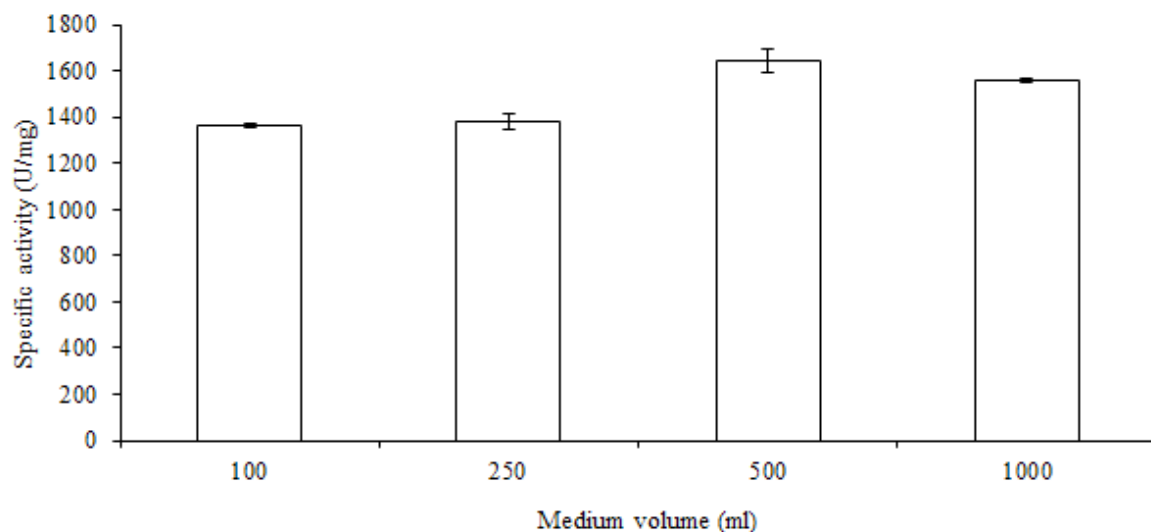


Figure 7. Effect of medium volume on the production of α -amylase by *B. licheniformis* ATCC 12759. Inoculum size 30% (by volume per mass), Moisture content 20%, Extraction medium with tap water, Initial pH 6.5, Incubation temperature 37°C, Incubation time 48.

The composition of rice bran is approximately 10% protein, 10% moisture, 20% crude fiber, 15% crude fat and 45% carbohydrates [38]. Maximum α -amylase production was obtained with rice bran as the substrate. It may not provide all the nutrients needed by the organism for maximum enzyme production or some of the vital nutrients necessary for growth and enzyme formation may be present at sub-optimal level. Hence, the exogenous addition of various nutrients to the solid medium improves the growth of organism and the product yield [39,40]. Addition of organic nitrogen sources such as casein, peptone, tryptone, yeast extract, beef extract, urea

and inorganic nitrogen source such as ammonium nitrate, sodium nitrate, ammonium chloride and ammonium sulphate to the medium were investigated. As shown in Table 3, in comparison with the control (1800.6 \pm 18.2 U/mg), there was increase in enzyme yield in the case of the supplementation of ammonium chloride (2094.9 \pm 53.1 U/mg) which proved to be the best among all the nitrogen sources. It has also been reported that nitrate was inferior to ammonia in α -amylase production [28,41].

Various inorganic salts were also studied for their effect on the synthesis and secretion of

Table 2. Effect of carbon sources on the production of α -amylase by *B. licheniformis* ATCC 12759. Inoculum size 30% (by volume per mass), volume of medium 100 ml, Extraction medium with tap water, Initial pH 6.5, Incubation temperature 37°C, Incubation time 48 h.

Carbon source (1%)	Specific Activity (U/mg)
Control*	1800.6 \pm 18.2
Mannose	829.5 \pm 47.1
Arabinose	1359.8 \pm 49.5
Sucrose	1414.4 \pm 20.9
Glucose	1725.1 \pm 18.9
Galactose	1244.9 \pm 48.7
Fructose	991.6 \pm 57.6
Lactose	1338.6 \pm 31.6
Xylose	218.6 \pm 18.3

*Control contains only rice bran and tap water.

Table 3. Effect of nitrogen sources on the production of α -amylase by *B. licheniformis* ATCC 12759. Inoculum size 30% (by volume per mass), volume of medium 100 ml, Extraction medium with tap water, Initial pH 6.5, Incubation temperature 37°C, Incubation time 48 h.

Nitrogen source (1%)	Specific Activity (U/mg)
Control*	1800.6 \pm 18.2
Sodium nitrate	1261.0 \pm 34.9
Ammonium sulphate	1888.1 \pm 28.4
Ammonium nitrate	1403.8 \pm 19.1
Ammonium chloride	2094.9 \pm 53.1
Beef extract	1076.0 \pm 8.7
Tryptone	1197.6 \pm 19.8
Peptone	1115.4 \pm 71.3
Yeast extract	1122.0 \pm 37.8
Urea	1174.0 \pm 80.0
Casein	842.6 \pm 19.7

*Control contains only rice bran and tap water.

Table 4. Effect of metal salt sources on the production of α -amylase by *B. licheniformis* ATCC 12759. Inoculum size 30% (by volume per mass), volume of medium 100 ml, Extraction medium with tap water, Initial pH 6.5, Incubation temperature 37°C, Incubation time 48 h.

Metal salts source (0.1%)	Specific Activity (U/mg)
Control*	1800.6±18.2
MgSO ₄	1730.6±28.5
ZnSO ₄	47.5±2.3
CaCl ₂	1763.1±69.7
CuSO ₄	54.7±30.9
FeSO ₄	1693.0±9.2

*Control contains only rice bran and tap water

α -amylase production. Comparison with the control (1800.6±18.2 U/mg), the production of α -amylase by *B. licheniformis* ATCC 12759 was suppressed by all metal salt sources (Table 4). The salt requirement for production of this particular enzyme was apparently provided by the nature of rice bran. These are important in terms of the cost of production of enzyme.

CONCLUSION

Solid waste from rice bran used as the basic nutrient source. Supplementation with carbon and metal salt sources decreased the enzyme production. Certain fermentation parameters involving incubation time, incubation temperature, inoculum level, moisture level, extraction medium, initial pH and medium volume were studied separately. Maximal amount of α -amylase production (2094.9±53.1 U/mg) was obtained inoculum level 30%, moisture level 20%, initial pH 6.5 at 37°C for 48 h with supplementation of ammonium chloride. Commercial α -amylase production is usually produced by submerged fermentation; however, SSF appear promising due to the natural potential and advantages they offer. Based on the present study, it appears that rice bran, which is inexpensive and readily available agricultural substance, could replace the commercial and more expensive substances in the development of a suitable economic fermentation medium for obtaining high yields of α -amylase. However, the present study was entirely a laboratory-scale study, and it has to be further improved for a large-scale SSF.

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