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Thermostable α-amylase from Lignocellulosic Residues Using *Bacillus amyloliquefaciens*

N. Noshadi, M. Mohammadi, G. D. Najafpour*, F. Pouryafar

Department of Chemical Engineering, Babol Noshirvani University of Technology, Babol, Iran

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ABSTRACT

In this study, thermostable high performance α-amylase was synthesized from lignocellulosic residues using *Bacillus amyloliquefaciens*. For this purpose, hydrolysates of wheat bran, rice bran and sugarcane bagasse were used as substrate for enzyme production. The maximum enzyme production was achieved in the medium containing hydrolysate of wheat bran. In order to enhance α-amylase production, the medium composition was optimized in terms of supplementary carbon and nitrogen sources. Enzyme activity in the optimized medium (208.94 U mL^{-1}) was considerably higher as compared to non-optimized medium (76.22 U mL^{-1}). The activity and stability of the synthesized enzyme was assessed in various temperature and pH environments. The optimum condition for highest enzyme activity (pH 7 and 70 °C) and stability (pH 7 and 50 °C) was determined. The effect of various metal ions on the α-amylase activity was investigated. The enzyme activity enhanced in the presence of Mg, Mn, Zn, Na, Cu, Ca ions, while Fe ion hindered the enzyme activity.

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1. INTRODUCTION

α-Amylases (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) are extracellular endoenzymes which catalyze the hydrolysis of internal 1,4-α-D-glucosidic linkages in starch, glycogen and various polysaccharides to smaller products such as glucose, maltose and maltotriose units [1-5]. Amylases account for approximately 25–30% of the world's enzyme market [4, 6, 7]. This enzyme is extensively used in different industrial fields like starch liquefaction, fermentation, pharmaceutical, food, paper, textile, detergent and sugar industries [4, 8].

Although α-amylases have been extracted from several sources such as plants, animals and microorganisms, however, microbial enzymes mainly from fungal and bacterial sources are used for industrial applications due to advantages like consistency, thermostability, cost effectiveness, reduced time and space required for production, high productivity and ease of process modification and optimization [2, 9, 10]. Although α-amylases can be produced by various strains of microorganisms, yet for commercial purposes, α-amylases are extracted from a few species of mesophilic fungi, mainly *Aspergillus* and *Penicillium* and from several species of genus *Bacillus* [4, 11, 12]. Microbial amylases due to advantages such as specificity of the reaction, elimination of neutralization steps, stability of the generated products and lower energy requirements, have successfully replaced acid hydrolysis process in more than 75% of starch hydrolyzing processes in industries [3, 9].

α-Amylase can be produced by two major techniques; submerged fermentation (SmF) and solid state fermentation (SSF). Although SmF has low productivity, but because of better control of the environmental and cultural conditions, has long been used on industrial scale [8, 13]. The major inefficiency in the production of α-amylase by SmF is the use of very expensive and uneconomical substrates in the fermentation media, including soluble starch, nutrient broth, etc. [13]. The utilization of agro-industrial residues could be a suitable route for the reduction of production cost of α-amylase [8, 14].

*Corresponding Author's Email: najafpour@nit.ac.ir (G. D. Najafpour)

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Several works describe the production of *α*-amylase using *Bacillus amyloliquefaciens* in media containing agro-byproducts like wheat barn, groundnut oil cake and corn bran in various fermentation processes [15]. However, so far there are very few reports on the use of agro-byproducts hydrolysate for the production of *α*-amylase using *B. amyloliquefaciens* in SmF. In this study, the production of *α*-amylase using *B. amyloliquefaciens* strain PTCC1732 in medium containing agro-byproducts hydrolysate such as wheat barn, sugarcane bagasse and rice barn is described, which is among the first reports of this method for *α*-amylase production by *B. amyloliquefaciens*. The fermentation condition was optimized in terms of incubation time, temperature, pH, carbon source and nitrogen source to achieve high yields of enzyme. The activity and stability of the synthesized enzyme in several environments is also investigated.

2. MATERIALS AND METHODS

2. 1. Preparation of Lignocellulosic Agricultural Wastes Hydrolysate Agricultural wastes including sugarcane bagasse, wheat bran and rice bran were obtained from local mills. These lignocellulosic residues were washed with distilled water, oven dried at 80 ˚C for 48 h and chopped in a mill (Moulinex, A320, Spain) to small particles. The ground biomass materials (20 g) were separately poured in the flasks containing 200 mL 0.25% (v/v) H_2SO_4 solution and then the mixtures were autoclaved at 121 ˚C for 60 min. The solid particles were then removed by filtration using Whatman No. 1 filter paper and the pH of the filtrate was adjusted to 7.0 using 1 M NaOH solution.

2. 2. Microorganism and Inoculum Preparation *Bacillus amyloliquefaciens* PTCC 1732, was obtained from the Iranian Research Organization for Science and Technology (IROST). The stock culture was maintained on nutrient agar slant at 4 ˚C. The seed culture was prepared by transferring a loop-full of bacteria from the agar slant into an Erlenmeyer flask containing 50 mL of medium consisting of (g L^{-1}): glucose, 10; yeast extract, 5; peptone, 5; KH_2PO_4, 1 and $MgSO_4$, 0.2; all chemical and biochemical were provided by Merck, Germany. The cells were incubated at 37 ˚C in a rotary shaker incubator (IKA, Ks 4000i control, Germany) at 130 rpm for 16 h.

2. 3. Enzyme Production Medium The enzyme production was carried out in the medium containing (g L^{-1}): yeast extract, 5; peptone, 5; KH_2PO_4, 1; $MgSO_4$, 0.2 and the hydrolysate obtained from the lignocellulosic agricultural residues (as described in Sec. 2.1) as the carbon source. The media for enzyme production were sterilized at 121 ˚C for 15 min. After cooling down to the room temperature, the media were inoculated with 3 v/v % of seed culture and incubated at 37 ˚C in a rotary shaker incubator (130 rpm) for different incubation times (24-96 h). Our preliminary tests indicated that the optimum incubation time for *α*-amylase production was 72 h. At prolonged incubation time beyond this period, the enzyme activity declined gradually, which could be attributed to the accumulation of other by-products in the medium because of the release of intracellular fractions of the cell at the end of the stationary phase due to cell lysis [16]. Based on the discussed results, all fermentation tests were terminated at 72 h. At the end of incubation period, cells were removed by centrifugation (5,000 rpm, 20 min) and the supernatant was used to measure *α*-amylase activity.

2. 4. *α-Amylase* Activity Assay The activity of *α*-amylase was assayed with soluble starch as substrate. The reaction mixture, containing 1 mL of soluble starch (1% w/v) solution made in 0.1 M phosphate buffer (pH 7) and 100 µL of the cell-free supernatant as the source of the crude enzyme was incubated at 50 ˚C for 5 min. The amount of reducing sugar liberated in the reaction was determined by 3,5-dinitrosalicylic acid (DNS) method using glucose as the standard [17]. One unit of *α*-amylase activity was defined as the amount of enzyme required to produce 1 µmoL of reducing sugar per minute from soluble starch under assay condition (50 ˚C and pH 7) and expressed as U mL^{-1} substrate. The scheme for production of enzyme from hydrolysate of lignocellulosic biomass is presented in Figure 1.

2. 5. Medium Optimization for Maximum *α*-Amylase Production

2. 5. 1. Effect of Lignocellulosic Agricultural Residues Hydrolysate The production of *α*-amylase was investigated in various media prepared with different hydrolysate solutions obtained from wheat bran, rice bran and sugarcane bagasse. The volumes of hydrolysate solutions in the media were calculated so that the initial sugar concentration was equal in all media.

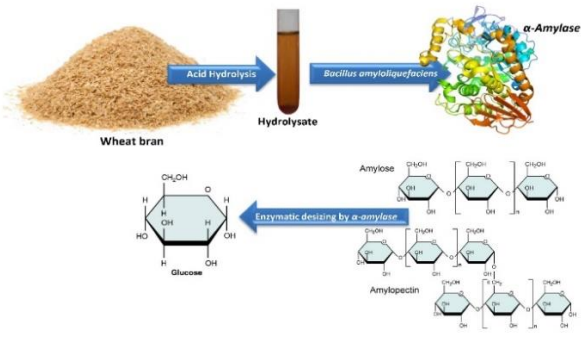

Figure 1. Scheme for production of enzyme from hydrolysate of lignocellulosic biomass

2. 5. 2. Effect of Supplementary Carbon and Nitrogen Sources The influence of various carbon supplements (2 g L^{-1}) including glucose, lactose, arabinose, fructose, rice bran hydrolysate, sugarcane bagasse hydrolysate and starch on *α*-amylase production was studied. Moreover, the effect of several nitrogen sources (1% w/v) such as peptone, yeast extract, ammonium chloride, sodium nitrate, corn husk, corn meal, soybean meal and urea on *α*-amylase production was investigate.

2. 6. Enzyme Characterization (α-amylase Activity and Stability)

2. 6. 1. Effect of Temperature To study the effect of temperature on the enzyme activity, the activities were examined at various temperatures between 30 and 90 ˚C for 5 min at pH 7.5. Thermal stability of the enzyme was investigated by incubating the enzyme at various temperatures (50 to 100 ˚C) for 60 min, then the relative enzyme activities were determined as the percentage of the crude enzyme activity, considered as 100%, measured under optimum enzyme assay condition.

2. 6. 2. Effect of pH The optimum pH for enzyme activity was determined by examining various pH levels (pH 4–13). In order to obtain the desired pH values, the following buffer solutions (0.1 M) were used: sodium acetate buffer (pH 4–5), potassium phosphate buffer (pH 7), Tris–HCl (pH 9), sodium carbonate–bicarbonate (pH 11) and KCl–NaOH buffer (pH 13). For pH stability, the enzyme solution was pre-incubated in various buffer solutions (pH 4–13) at 30 ˚C for 30 min and the relative enzymatic activities were calculated under optimum conditions of pH as described earlier.

2. 6. 3. Effect of Metal Ions To investigate the effect of different metal ions (Ca, Mg, Na, Fe, Mn, Zn and Cu) on the enzyme activity, the *α*-amylase was individually pre-incubated with 10 mM solution of various metal ions ($CaCl_2.2H_2O$, $MgCl_2.6H_2O$, NaCl, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, $ZnSO_4$ $.7H_2O$ and $CuSO_4.5H_2O$) at 70 ˚C for 1 h and the relative enzyme activity was calculated.

For the sake of ensuring repeatability of the results, all experiments were repeated at least two times and results were reported as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3. 1. Optimization Results for Enzyme Production Among the physicochemical parameters, pH of the growth medium plays an prominent role in enzyme production [7]. Studies have shown that most of *Bacillus* strains commercially used for the production of *α*-amylases in SmF have an optimum pH between 6 and 7 for growth and enzyme secretion [18, 19]. In our experimental runs, pH 7 was used in all fermentation media. Incubation temperature is also well known as an influential parameter on cell growth and production of enzymes or other metabolites is usually sensitive to the medium temperature [16]. Microbial *α*-amylases are mostly secreted as the primary metabolites and their production is growth associated [20]. Thus, it could be assumed that the temperature of 37 ˚C, which was recommended by IROST as the optimum temperature for cell growth, was suitable to achieve the highest enzyme production. As a proof of concept, some preliminary tests were carried out considering the influence of temperature (33-45 ˚C) on the production of enzyme. The obtained results confirmed our speculation where the optimum temperature for cell growth and *α*-amylase production was 37 ˚C. As the incubation temperature increased to beyond 37 ˚C, the culture turbidity (OD reading) decreased and the enzyme production reduced. High incubation temperature could lead to changes in the cell membrane composition and the protein catabolism [21]. It is also most probable that high temperatures imposed inhibition on the cell growth and thus hindered the enzyme production.

Considering the above mentioned discussion, pH 7 and temperature of 37 ˚C were considered as the constant fermentation condition and the medium optimization was carried out to enhance the enzyme production.

3. 1. 1. Effect of Lignocellulosic Biomass Hydrolysate Figure 2 depicts the influence of different lignocellulosic biomass hydrolysates used as carbon source on the *α*-amylase production. As shown in this Figure, the maximum enzyme was produced in the presence of wheat bran hydrolysate with the activity of 76.2 U mL^{-1}. Wheat bran is an inexpensive agricultural residue rich in nutrients such as proteins, various amino acids, carbohydrates and different metal ions that can stimulate the growth of microorganisms. All these nutrients are necessary for cell biomass formation as well as enzyme production [21].

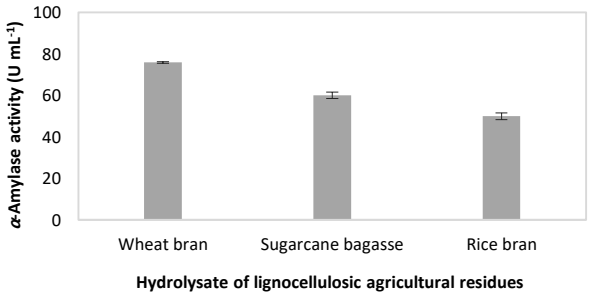

Figure 2. Effect of lignocellulosic biomass hydrolysate on the *α*-amylase production

Considering that wheat bran hydrolysate produced the highest amount of enzyme among the studied lignocellulosic biomass residues, it was used as carbon source in the medium in the subsequent optimization experiments.

3. 1. 2. Effect of Supplementary Carbon Sources and Nitrogen Sources The influence of supplementary carbon source such as glucose, lactose, arabinose, fructose, rice bran hydrolysate, sugarcane bagasse hydrolysate and starch at the concentration of 2 g L^{-1} on the enzyme production was investigated; the results are reflected in Figure 3. The highest enzyme production was achieved with glucose and sugarcane bagasse hydrolysate (176.7 U mL^{-1}). 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 [22]. Based on this knowledge and considering that sugarcane bagasse hydrolysate performed as efficient as glucose to provide carbon and energy in the medium, the hydrolysate of this abundant lignocellulosic waste was used as the carbon supplement in the subsequent optimization experiments. The concentration of this carbon supplement in the medium was optimized by varying its concentration in the range of 1-5 g L^{-1}; the results are illustrated in Figure 4. As results show, the highest α-amylase activity (208.98 U mL^{-1}) was achieved while the concentration of sugarcane bagasse hydrolysate in the medium was 4 g L^{-1}.

Both the nature and relative concentration of different complex nitrogen compounds in the medium are important in the synthesis of α-amylase. Low levels of nitrogen and also excess amount of nitrogen are equally detrimental, causing enzyme inhibition [23]. The influence of several inorganic and organic nitrogen sources on α-amylase production was examined and the results are depicted in Figure 5. It was interesting to see that there was considerable decrease in the enzyme production in case of supplementation of medium with either inorganic or organic nitrogen sources in comparison with the control medium, wherein the nitrogen was provided by a combination of peptone and yeast extract.

It was also attempted to find the best concentration of peptone and yeast extract as the nitrogen supplements in the medium. For this purpose, the concentration of these compounds, which were used at the ratio of 1:1, was varied in the range of 1 to 6 g L^{-1} (Figure 6). The results showed that maximum α-amylase production was obtained at the concentration of 5 g L^{-1} of nitrogen compounds.

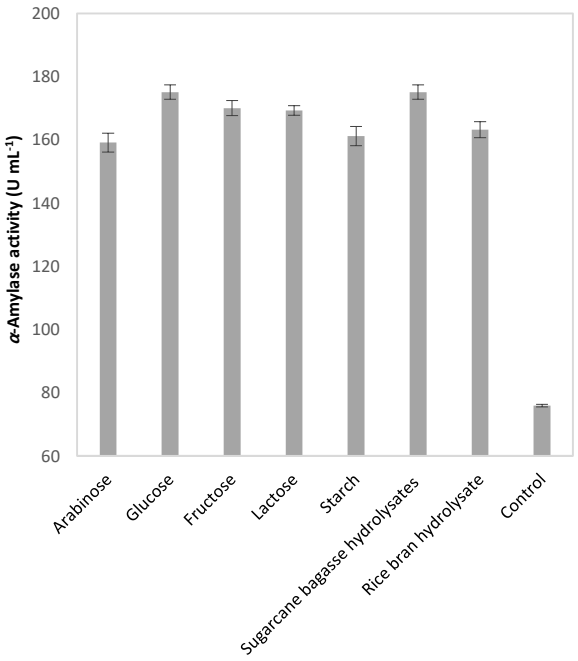

Figure 3. Effect of supplementary carbon source (2 g L^{-1}) on the α-amylase production

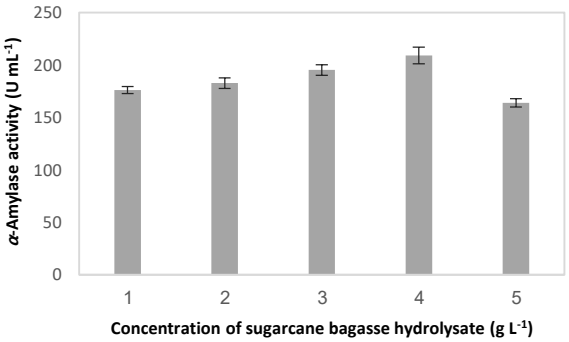

Figure 4. Effect of concentration of sugarcane bagasse hydrolysate on the α-amylase production

3. 2. Optimization Results for Enzyme Activity and Stability

3. 2. 1. Effect of Temperature The influence of temperature on the activity and stability of the α-amylase enzyme was studied in phosphate buffer solution (0.1 M) at different temperatures in the range of 30 to 90 ˚C and pH 7.5. Figure 7 illustrates the effect of temperature on the activity and stability of the enzyme. As results show, the enzyme was active in a wide range of temperatures, between 30 and 90 ˚C, with optimum activity at 70 ˚C; however, a reduction in the enzyme activity was observed at the temperatures above 70 ˚C.

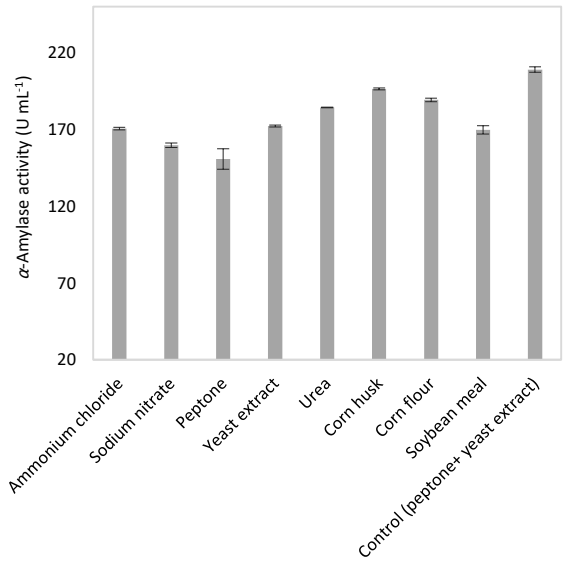

Figure 5. Effect of nitrogen sources on the α-amylase production

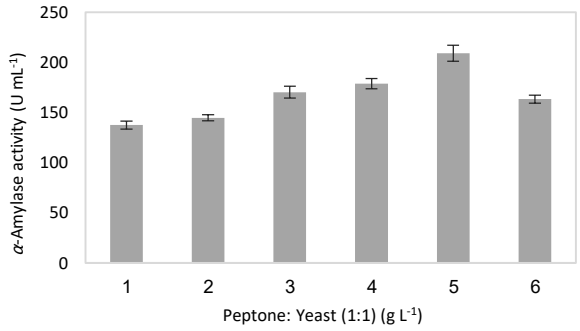

Figure 6. Effect of concentrations of yeast extract and peptone on α-amylase production

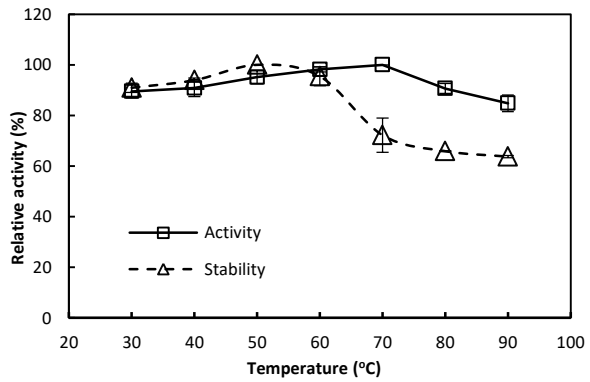

Figure 7. Effect of temperature on activity and stability of the α-amylase from *B. amyloliquefaciens*. The enzyme activity was measured after 5 min incubation in water bath and the stability was determined after 60 min incubation in phosphate buffer solution (0.1 M) at pH 7.5

Such behavior might be attributed to the fact that, as the temperature increases, the enzyme starts to deactivate progressively due to thermal inactivation of proteins, inappropriate conformation of protein, hydrolysis of the peptide chain and destruction or aggregation of amino acids [24].

Stability of the enzymes is a desirable property for their industrial application [7, 23]. Therefore, thermostability of the synthesized enzyme was assessed by incubating the enzyme for 60 min at different temperatures in the range of 30 to 90 °C. The stability behavior of the enzyme is presented in Figure 7. As observed in this Figure, the enzyme showed high thermal stability and retained more than 90% of its original activity in the temperature range of 30 to 60 °C; nevertheless, the enzyme stability decreased significantly at the temperatures above 60 °C. This phenomenon could be due to the denaturation of enzyme at high temperatures [25].

3. 2. 2 Effect of pH Figure 8 shows the influence of pH (4-13) on the activity and stability of the synthesized α-amylase. The α-amylase had appropriate activity between pH 4 and 11; more than 80% of enzyme activity was retained in this pH range, with maximum activity at pH 7. These results could be expected as α-amylases from most bacteria and fungi are generally known to be active at acidic or neutral pHs [18].

The pH stability of the enzyme was examined by measurement of the relative activity after incubation at different pHs at 30 °C for 30 min. As results in Figure 8 show, the enzyme had excellent stability in the pH range of 5 to 9 and retained more than 70% of its original activity after 30 min incubation in this pH range. However, the enzyme activity decreased at pHs below 5 and above 9.

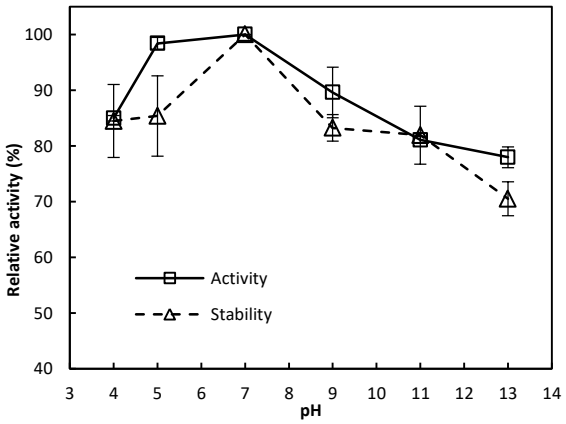

Figure 8. Effect of pH on activity and stability of the α-amylase from *B. amyloliquefaciens.* The enzyme activity and stability were respectively measured after 5 and 30 min incubation in different buffers at 30 °C

This shows that the synthesized enzyme could not be used at strong acidic or high alkaline conditions.

3. 2. 3. Effect of Metal Ions on Enzyme Activity Metal ions play an important role as cofactors for enzyme activity and often act as salt or ion bridges between two adjacent amino acid residues [26]. A number of enzymes require the presence of metal ions, such as calcium ions, for the maintenance of their stable and active structures [27]. In this study, the effect of different metal ions on enzyme activity was examined. As shown in Table 1, the enzyme activity increased in the presence of Mn, Mg, Zn, Na, Cu and Ca ions; especially Mn and Mg considerably enhanced the enzyme activity. However, the activity of the enzyme was inhibited by Fe ion. The inactivation of the enzyme in the presence of Fe ion may be due to competition between the exogenous cations and the protein-associated cations in the active site of the enzyme which resulted in decreased metalloenzyme activity [25, 28].

TABLE 1. Effect of metal ions on the activity of α-amylase

Metal ions (10 mM)	Relative activity (%)
$MnCl_2.4H_2O$	120.72 ± 0.62
$MgCl_2.6H_2O$	118.35 ± 0.95
$ZnSO_4 .7H_2O$	106.27 ± 0.29
NaCl	105.6 ± 3.25
$CuSO_4.5H_2O$	105.6 ± 6.49
$CaCl_2.2H_2O$	102.6 ± 8.76
$FeSO_4.7H_2O$	94 ± 5.65

3. 3. Comparison between the Results of this Work and Other Researches The ability of various bacterial species for nutrient uptake in the medium is different, therefore they represent different cell growth and enzyme production. So far, there are very few reports on the use of agro-byproducts hydrolysate for the production of α-amylase from different microbial species, especially *B. amyloliquefaciens* in submerged fermentation. This study is among the first works which report the production of α-amylase from hydrolysate of agricultural residues using *B. amyloliquefaciens* in SmF. A comparison between the outputs of the present work and other reports available in the literature is summarized in Table 2. The produced α-amylase showed favorable activity, comparable with other works. The fermentation was performed under mild operation condition and low cost lignocellulosic residues were used as carbon source which can add to the advantages of this work.

TABLE 2. Comparison between the results of the present work and other researches for α-amylase production in SmF

Substrate	Microorganism	Fermentation conditions	Activity (U mL-1)	Reference
Sugarcane bagasse hydrolysate	*Aspergillus niger NCIM 548*	pH: 5.65 Incubation time: 76.67 h T: 30 °C	57.24	[29]
Sago starch hydrolysate	*Bacillus subtilis* SSII2	pH:7 Temperature: 32 °C Incubation time: 12 h	538	[19]
Sugarcane bagasse hydrolysate	*Bacillus subtilis*	Temperature: 37 °C Incubation time: 30-36 h	144	[1]
Corn flour	*Bacillus amyloliquefaciens*	pH: 9 Temperature: 42 °C Incubation time: 48 h	54.93 ± 0.18	[23]
Synthetic medium	*Anoxybacillus flavithermus*	pH: 7 Temperature: 55 °C Incubation time: 24 h	1509.3	[28]
Waste potato starch	*Bacillus subtilis* JS-2004	pH: 7 Temperature: 50 °C Incubation time: 48 h	72	[7]
Rice bran hydrolysate	*Bacillus amyloliquefaciens*	pH:7 Temperature: 37 °C Incubation time: 72 h	208.94 ± 5.12	This work

4. CONCLUSIONS

In this work, the production of α-amylase from the hydrolysate of some lignocellulosic residues using *B. amyloliquefaciens* was investigated. The results showed that wheat bran hydrolysate was suitable substrate for high yield α-amylase production in submerged fermentation. To achieve high enzyme production, the fermentation medium was optimized in terms of carbon supplement and nitrogen source. The activity of the enzyme in the optimized medium was 2.75 times that of non-optimized one. The synthesized α-amylase displayed wide pH stability and moderate thermostability. These features indicate that this enzyme can be useful for usage in starch processing and detergent industries.

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Thermostable *α*-amylase from Lignocellulosic Residues Using *Bacillus amyloliquefaciens*

N. Noshadi, M. Mohammadi, G. D. Najafpour, F. Pouryafar

Department of Chemical Engineering, Babol Noshirvani University of Technology, Babol, Iran

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چکیده

در این پژوهش، آنزیم آلفا–آمیلاز مقاوم در برابر حرارت با استفاده از ضایعات لیگنوسلولزی توسط باکتری باسیلوس آمیلولیکوفسینس تولید شد. برای این منظور، از محلول حاصل از هیدرولیز سبوس گندم، سبوس برنج و تفاله نیشکر به عنوان سوبسترا برای تولید آنزیم استفاده گردید. بیشترین میزان تولید آنزیم در محیط کشتی بود که حاوی عصاره هیدرولیز سبوس گندم بود. برای افزایش میزان تولید آلفا–آمیلاز، محیط کشت از نظر میزان مکمل‌های کربنی و نیتروژنی بهینه‌سازی شد. فعالیت آنزیمی در محیط کشت بهینه (۲۰۸/۹۴ واحد بر میلی‌لیتر) بسیار بالاتر از محیطی بود که بهینه‌سازی در آن انجام نشده بود (۷۶/۲۲ واحد بر میلی‌لیتر). فعالیت و پایداری آنزیم سنتز شده در محیطهای مختلف با دما و pH متفاوت بررسی شد. شرایط بهینه برای داشتن بیشترین فعالیت آنزیمی (pH برابر با ۷ و دمای ۷۰ درجه سانتی‌گراد) و بالاترین پایداری (pH برابر با ۷ و دمای ۵۰ درجه سانتی‌گراد) تعیین شد. تاثیر حضور یونهای فلزی مختلف روی فعالیت آنزیمی بررسی گردید. فعالیت آنزیمی در حضور یونهای منیزیم، منگنز، روی، سدیم، مس و کلسیم بهبود یافت در حالی که یون آهن فعالیت آنزیمی را کاهش داد.

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