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SYNERGISTIC EFFECTS OF AGRO-INDUSTRIAL WASTES ON ALPHA AMYLASE PRODUCTION BY BACILLUS AMYLOLIQUEFACIENS IN SEMI SOLID SUBSTRATE FERMENTATION

YARI KATI HAL FERMENTASYONUNDA BACILLUS AMYLOLIQUEFACIENS İLE ALFA AMİLAZ ÜRETİMİNE TARIMSAL ATIKLARIN SİNERJİK ETKİLERİ

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Abstract

This study concerns with the combinations of soybean meal (SM), wheat bran (WB) and whey were utilized as the substrates for α-amylase production by Bacillus amyloliquefaciens NRRL B-645. As a result of the experiments, the highest α-amylase activity (4257 U/ml) was obtained containing SM 20 g/l, WB 5 g/l, whey 5% (v/v), peptone 1 g/l, yeast extract (YE) 0.5 g/l and $(NH_4)_2SO_4$ 1 g/l at 33 °C and 150 rpm for 48 h. This innovative process for the α-amylase production can be extended to different enzymes using various agro-industrial wastes.

Keywords: Alpha amylase, *Bacillus amyloliquefaciens NRRL B-645*, Agro-industrial wastes, Semi solid substrate fermentation.

Öz

Bu çalışma; Bacillus amyloliquefaciens NRRL B-645 ile substrat olarak soya küspesi (SM), buğday kepeği (WB) ve peynir altı suyu kombinasyonları kullanılarak α-amilaz üretimi ile ilgilidir. Deneyler sonucunda en yüksek enzim aktivitesi SM 20g/l, WB 5g/l, peynir altı suyu 5% (v/v), peptone 1 g/l, yeast ekstrakt (YE) 0.5 g/l ve $(NH_4)_2SO_4$ 1 g/l konsantrasyonunda 48 saat sonunda, 33 °C fermantasyon sıcaklığı, 150 devir/dk karıştırma hızında 4257 U/ml olarak elde edildi. α-amilaz üretimi için kullanılan bu yeni proses, farklı tarımsal atıklar kullanılarak farklı enzimlerin üretiminde de kullanılabilir.

Anahtar kelimeler: Alfa Amilaz, *Bacillus amyloliquefaciens NRRL B-645*, Tarımsal atıklar, Yarı katı hal fermantasyonu.

1 Introduction

Alpha amylases are extremely important enzymes and extensively used in various industries such as biotechnology, food, detergent, textile and paper industries. They can be isolated from living organisms such as animals, plants, microorganisms. However, the microorganisms are generally preferred for alpha amylase production due to their ability to withstand extreme environmental conditions [1],[2]. The most abundantly used α-amylases of microbial origin are derived from *Bacillus amyloliquefaciens, B. licheniformis and B. stearothermophilus* [3].

The production of α-amylases used in the above mentioned industries is commonly obtained by the submerged fermentation (SMF), but solid substrate fermentation (SSF) has some positive aspects for commercial applications. In this work, the SSF system was modified as semi solid substrate fermentation (SSSF), by increasing the water content in order to increase nutrient availability by microorganism and to enable control of fermentation parameters [4],[5]. It was described as the insoluble substrate in which a solid porous matrix, absorbs water with a relatively high water activity and substrate also contains available the other components [6]. This process provided easier growth and higher efficiency of enzyme production due to high water activity, which is more efficient method than the solid state one [7].

It is known that the mainly carbon sources such as starch and glucose are not advantageous for α-amylase production because of their highly costs. Therefore, the studies are progressed about cheaper carbon sources. Many researchers in previous studies have been studied the effects on α-amylase production of the cheap and easily accessibility agro-industrial wastes such as wheat straw, wheat bran, coffee waste, banana waste, potato peel and sugarcane bagasse, soybean meal, rice bran, maize oil cake, orange peel, apple peel [8]-[15]. However, most of these studies indicated α-amylase production using individual substrates. There is no much study in the literature deal with the synergistic effects of agro-industrial wastes on α-amylase production.

In this work, we present a novel approach related to the investigation the synergistic effects of different agro-industrial wastes for α-amylase production by *B. amyloliquefaciens NRRL B-645'*in SSSF.

2 Materials and Methods

2.1 Microorganism and Medium

Bacillus amyloliquefaciens NRRL B-645 was obtained from Agricultural Research Service Culture Collection in USA. The strain was maintained on slant at 4 °C. A standard growth medium containing glucose 15 g/l, peptone 2.5 g/l, YE 2 g/l, NaCl 1.5 g/l, KH_2PO_4 0.5 g/l, $MgSO_4$ 0.5 g/l and $CaCl_2$ 0.1 g/l was prepared into 250 ml. Erlenmayer flask which was then kept at 37 °C and 150 rpm for 18 h. The initial pH of the medium was adjusted to 7.0 and sterilized by autoclaving at 121 °C for 15 minutes. Inoculum 1% (v/v) was transferred into 250 ml

erlenmayer flasks containing 50 ml enzyme production medium [16].

The production medium contains only SM and tap water. However, in order to determine the effects of the different concentrations of medium constituents and process conditions on the production of α-amylase, the enzyme production medium described was prepared according to previous research of Anderson et al. [16]. The pH of medium was initially adjusted to 7.0 and was allowed to follow its natural course throughout the fermentation. All the experiments were conducted in triplicate and the data used in the figures are mean values of three experiments.

2.2 Determination of α-amylase Activity

The fermented broth was taken after 30 h and centrifuged at 7000 rpm for 15 minutes and then supernatant was used for α-amylase assay. The activity was measured by decrease in iodine color reaction showing dextrinization of starch. The activity was measured against to the control in which no enzyme was added. The reaction mixture contained 0.5 ml of enzyme and 4.5 ml substrate solution, which was incubated at 37 °C for 30 minutes. Substrate solution contained 2.3 ml starch (3%), 1 ml 0.1 N $CaCl_2$, 250 ml phosphate tampon (pH 6.2) containing 0.025 N NaCl and 200 ml distilled water. After incubation, the reaction was stopped by adding 0.9 ml 1 N HCl in both samples and 0.5 ml enzyme was added to enzyme-free sample, then 0.1 ml iodine solution (0.05% I_2, 0.5% KI) and 4 ml distilled water was added. Absorbance values were measured at 620 nm for both samples. One unit of enzyme activity is defined 0.0284 optical density reduction of blue color intensity of starch iodine solution at 37 °C [17]. All the experiments were performed independently from each other. The enzyme activities used for representations were the average values of three independent experiments.

3 Result

3.1 The Effect of Substrate Concentration on α-amylase Activity

It is well known that substrate concentration has direct effect on the water content of fermentation medium. The amount of water decreases with increasing substrate concentration in fermentation medium. When the water content is insufficient and does not allow to effective diffusion of solutes, the metabolism of microorganism gets down. Therefore, in order to assess the effect of substrate concentration on α-amylase activity, six different SM concentrations ranging from 5 to 50 g/l were used. As seen from Figure 1, the α-amylase activity increased with increasing SM concentration until 20 g/l; however any further increase in SM concentration, resulted in decrease on enzyme production.

The change of α-amylase activity with incubation time in fermentation medium containing only SM 20 g/l and tap water is shown in Figure 2. At the end of 48 h, α-amylase activity achieved a peak of 1572 U/ml and it relatively decreased on the next hours for this fermentation medium. This state may be attributed to the concentrations of essential nutrients are not found sufficiently in above mentioned medium conditions (Figure 2).

In order to assess the effect inoculum level on the α-amylase activity, various inoculum levels % (v/w) (1, 2.5, 5, 7.5, 10, 15, 20) were studied. The results show that there is not considerable change in activity values (data not given).

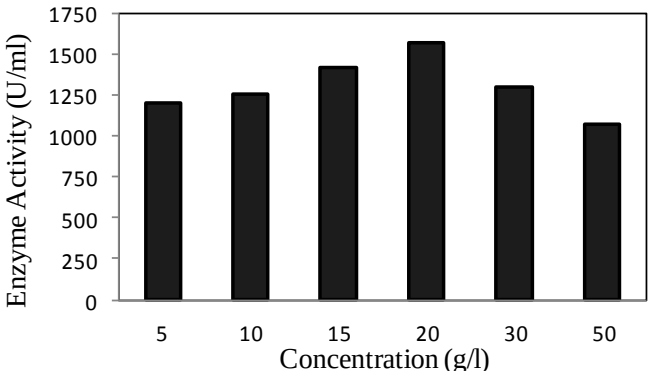

Figure 1: The effect of SM concentrations on α-amylase activity. (Initial pH: 7.0, incubation temperature: 37 °C, Agitation speed: 150 rpm).

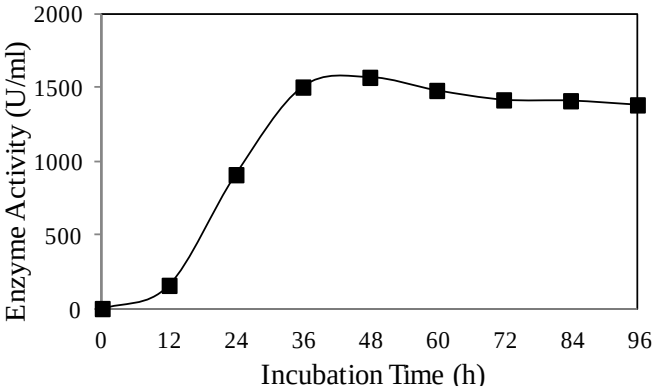

Figure 2: Time course α-amylase activity. (Initial pH: 7.0, SM concentration: 20 g/l, Incubation temperature: 37 °C, Agitation speed: 150 rpm).

In order to determine the effects of supplementation carbon sources such as corn protein (CP), wheat bran (WB), hazelnut oil cake (HOC) and whey on α-amylase activity, six different concentrations ranging from 5 g/l to 50 g/l were used individually. As seen from the Table 1, all the substrate combinations showed notable increase in the α-amylase activity and the highest activity (3018 U/ml) was achieved in medium containing SM 20 g/l and WB 5 g/l. The relationship between WB concentrations and enzyme activities is also presented in Table 2.

Table 1: The effect of supplementation carbon sources on α-amylase activity (Initial pH: 7.0, Incubation temperature: 37 °C, Agitation speed: 150 rpm).

Substrate combinations	Concentration (g/l)	Enzyme activity (U/ml)
SM + CP	20 + 10	2540
SM + WB	20 + 5	3018
SM + HOC	20 + 5	2905
SM + Whey	20 + 5% (v/v)	2954

3.2 The Effect of Supplementation Nitrogen Sources on α-amylase Activity

It is clearly known that the organic/inorganic nitrogen sources have significant influence on the enzyme activity in fermentation processes [18]. The effects of different concentrations (0.5, 1, 2.5, 5, 7.5, 10, 15, 20 g/l)

supplementation organic (YE, whey, peptone) and inorganic ($(NH_4)_2S0_4$, $NaNO_3$, NH_4NO_3, urea) nitrogen sources on α-amylase activity in fermentation medium containing SM 20 g/l and WB 5 g/l (without any nitrogen supplementation) were investigated individually. As seen from the Table 5, the maximum enzyme activity was obtained in the fermentation medium containing whey 5.0% (v/v), peptone 1 g/l, YE 0.5 g/l and $(NH_4)_2S0_4$ 2.5 g/l. Since the combinations of various substrates were used in this fermentation process, nitrogen requirement of microorganism could be met not only from carbon sources but from nitrogen-containing carbon sources too. At the light of these results; it is possible to emphasize that SM, WB and whey also can serve as nitrogen source in addition to being good carbon sources [19].

Table 2: The effect of WB concentrations in medium containing only SM 20 g/l and tap water on α-amylase activity (Initial pH: 7.0, Incubation temperature: 37 °C, Agitation speed: 150 rpm).

WB (g/l)	Enzyme activity (U/ml)
5	3018
10	2958
15	2810
20	2690
30	2570
50	2400

3.3 The Effect of Initial pH on α-amylase Activity

Incubation temperature is vital importance in SSSF since the metabolic heat generated during the fermentation process causes negative effects on α-amylase activity [20],[21]. In this study, five incubation temperatures (30, 33, 37, 40 and 45 °C) were carried out. As seen from the Table 3, the highest enzyme activity (4257 U/ml) was obtained at 33 °C in 48 h. When the incubation temperature is particularly increased up to 45 °C, the enzyme activity hugely declined (285 U/ml). This result may be attributed to reduction of the moisture contents in the fermentation medium at high temperatures [18].

Table 3: The effect of incubation temperature on α-amylase activity. (Initial pH: 7.0, Agitation speed: 150 rpm).

Temperature (°C)	Enzyme activity (U/ml)
33 (Control)	4241
30	3687
33	4257
37	4014
40	1273
45	285

3.4 The effect of Agitation Speed on α-amylase Activity

The α-amylase production is overly sensitive to initial pH of the fermentation medium [19]. The initial pH of medium was adjusted varied from 5.0 to 9.0 each at 1.0 interval after the sterilization in the autoclave, to study the effect of initial pH on enzyme activity. As seen from Table 4, the highest enzyme activity was (4257 U/ml) at the initial pH=7.0. According to these results, enzyme activity is generally stable from pH=5.0 to 9.0, thus indicating an exceptional buffering feature of the agro-industrial wastes as shown in Figure 3.

3.5 The Effect of Agitation Speed on α-amylase Activity

The enzyme production in fermentation process is susceptible to mechanical force, which may disturb the elaborate shape of complex molecule to such a degree that denaturation occurs [22]. Likewise, different agitation speeds in the enzyme production seemed to ensure different distribution of substrates, transition of required oxygen and nutrients to the microorganism [23]. In this context, α-amylase production was studied in five different agitation speeds (100, 125, 150, 175 and 200 rpm).

Table 4: The effect of initial pH on α-amylase activity (Incubation temperature: 33 °C, Agitation speed: 150 rpm).

pH	Enzyme activity (U/ml)
7.0 (Control)	4257
5.0	4110
6.0	4170
7.0	4241
8.0	4130
9.0	4200

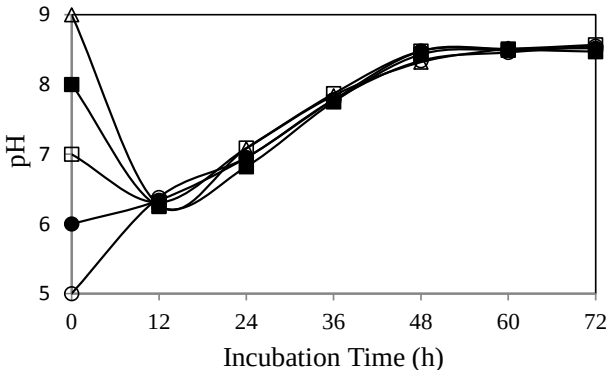

Figure 3: The effect of initial pH on α-amylase activity by *B. amyloliquefaciens NRRL B-645*. (○ pH: 5.0, ● pH: 6.0, □ pH: 7.0, ■ pH: 8.0, Δ pH: 9.0, Incubation temperature: 33 °C, Agitation speed: 150 rpm).

The highest α-amylase activity (4257 U/ml) was obtained at 150 rpm as seen from Figure 4. Any further increase in the agitation speed caused to decrease in α-amylase activity. Similarly, the enzyme activity was inhibited in lower agitation speeds. Since the growth of *B. amyloliquefaciens NRRL B-645* reduce at lower and higher agitation speeds than 150 rpm, the enzyme production also gets slow accordingly. Similar results were reported by previous researchers [22],[24],[25].

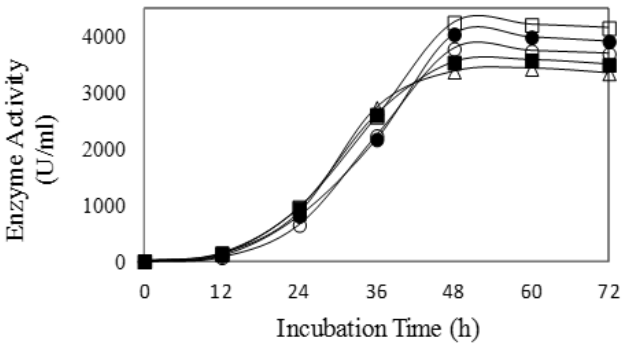

Figure 4: The effect of agitation speed on α-amylase activity (○ 100 rpm, ● 125 rpm, □ 150 rpm, ■ 175 rpm, Δ 200 rpm, incubation temperature: 33 °C, initial pH: 7.0).

Table 5: The effect of supplementation nitrogen sources on α-amylase activity (Initial pH: 7.0, Incubation temperature: 37 °C, Agitation speed: 150 rpm).

Combinations of nitrogen sources	Concentration (g/l)	Enzyme activity (U/ml)
Control (without any nitrogen supplementation)		3018
Whey	5 % (v/v)	3450
Whey + Peptone	5 % (v/v) + 1	3607
Whey + Peptone + YE	5 % (v/v) + 1 + 0.5	3710
Whey + Peptone + YE + $(NH_4)_2S0_4$	5 % (v/v) + 1 + 0.5 + 2.5	4078
Whey + Peptone + YE + $(NH_4)_2S0_4$+ Urea	5 % (v/v) + 1 + 0.5 + 2.5 + 0.5	3407
Whey + Peptone + YE + $(NH_4)_2S0_4$+ $NaNO_3$	5 % (v/v) + 1 + 0.5 + 2.5 + 0.5 +0.5	3893
Whey + Peptone + YE + $(NH_4)_2S0_4$+ NH_4NO_3	5 % (v/v) + 1 + 0.5 + 2.5 + 0.5 +0.5	3810

4 Conclusions

The following conclusions were achieved in this study concerning α-amylase production by *Bacillus amyloliquefaciens NRRL B-645* in SSSF. According to results of the experiments, the highest α-amylase activity (4257 U/ml) was obtained containing SM 20 g/l, WB 5 g/l, whey 5% (v/v), peptone 1 g/l, YE 0.5 g/l and $(NH_4)_2SO_4$) 1 g/l at 33 °C, and 150 rpm for 48 h. By the way, the future researchers can focus on the following topics;

1. The *Bacillus amyloliquefaciens NRRL B-645* can be modified genetically for increasing enzyme activity,
2. The different agro-industrial wastes can be used as carbon source to minimize the production cost of enzyme,
3. The residue solid wastes after the fermentation process have the high nutrional value and protein content can be considered as animal feed,
4. This innovative process for the α-amylase production also can be extended to various enzymes,
5. The present study was entirely a laboratory scale study, and it has to be further improved for a large scale SSSF.

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