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ALPHA-AMYLASE PRODUCTION IN SHAKE FLASK CULTURE OF *BACILLUS SUBTILIS* NRRLB3411

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ABSTRACT

Bacillus subtilis NRRL B3411 was found to give the highest yield of alpha-amylase in shake-flask culture in comparison with *Bacillus licheniformis* NRRL 1001 and a local isolate of *Bacillus amyloliquefaciens.* The cultural and nutritional requirements for alpha-amylase production by this organism were determined. Peptone was the ideal nitrogen source. Optimal values of the pH, temperature, and period of incubation for alpha-amylase formation were 6.5-7.5,40^{o}C, and five days, respectively.

INTRODUCTION

Alpha-amylase (∝-1,4 glucan, 4-glucanohydrolase, E.C. 3.2.1.1) is an endoenzyme which is able to dextrinize or 'thin' starch. This enzyme is commonly employed prior to starch saccharification by glucoamylase in the conversion of starch into glucose, high-fructose syrup or ethanol (1,2). It is an important industrial enzyme and its availability at low-cost is necessary for the large-scale processing of starch.

At present, the large-scale production of alpha-amylase in several countries is done by using selected *Bacillus* strains. Many of these strains have been selected and developed by industrial companies in order to produce an enzyme of high dextrinizing activity and thermal stability. Although many of these industrial strains are patented or covered by proprietary restrictions, some are obtainable from culture collections as well as local microbial isolates which have acceptable characteristics for alpha-amylase production.

The present paper deals with the preliminary evaluation of three *Bacillus* species for the production in shake-flask culture of alpha-amylase. The three species were compared in terms of enzyme productivity. Enzyme production by the most productive species was further studied in terms of medium composition, pH and temperature.

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MATERIAL AND METHODS

A. Comparison of Microorganisms.

Organisms. *Bacillus subtilis* NRRL B3411 was obtained from the Northern Regional Research Center, Peoria, Illinois, U.S.A. *Bacillus licheniformis* NRRL 1001 was given by Dr. A.K. Raymundo of BIOTECH-UPLB and a local isolate of *Bacillus amyloliquefaciens* was donated by Dr. S.C. Halos of FORI-BIOTECH.

The organisms were maintained by weekly transfers into TGYA (tryptone-glucose-yeast extract-agar) slants with the following composition (g/l): Tryptone, 5; glucose, 1; yeast extract, 3; agar, 25; and distilled water.

Media. Alpha-amylase production by *B. subtilis* and *B. amyloliquefaciens* was compared using a control medium (3) with the following composition (g/1): cassava root powder, 20; K_2HPO_4, 1; yeast extract, 5; peptone, 10; $MgSO_4$, $7H_2O$, 0.2; and distilled water. In the comparison of alpha-amylase production by *B. subtilis* and *B. licheniformis,* the yeast extract in the control medium was replaced by rice bran (18 g/1). The pH of both media was adjusted to pH 7.0 with a Radiometer pH M26 pH meter using either 85% H_3PO_4 or 3 N NaOH. The media were sterilized at 121°C (15 psig) for 15 minutes in a pressure cooker.

Inoculum Build-up. The inoculum for enzyme production was prepared by transferring four loopfuls of culture from fresh TGYA slants into 100 ml of medium in 250-ml Erlenmeyer flasks. The cultures were incubated for 24 hours in a rotary shaker. In the experiment using *B. subtilis* and *B. amyloliquefaciens,* incubation was made at room temperature (~ 29°C) while for the experiment using *B. subtilis* and *B. licheniformis,* the flasks were incubated in an Eberbach water bath-shaker at 40°C.

Enzyme Production. Five ml of the 24-hour old inoculum were transferred into several Erlenmeyer flasks, each containing 45 ml of medium. An initial cell count was made using the plate count technique (4).

In the experiment using *B. subtilis* and *B. amyloliquefaciens,* the cultures were incubated in a rotary shaker at room temperature (~29°C). Flasks were taken each day for the enzyme assay for a period of one week.

In the experiment using *B. subtilis* and *B. licheniformis,* the cultures were incubated in the Eberbach water bath shaker at 40°C. Flasks were taken after 3, 5, and 6 days of incubation.

Determination of the Optimum Temperature for Enzyme Production by B. licheniformis. The medium used for this experiment was the same as the medium used for comparing enzyme production by *B. subtilis and B. licheniformis.* Incubation of the build-up flask and of the flasks for enzyme production was made in the Eberbach water bath shaker at 50°C. Flasks were taken daily for the enzyme assay for a period of six days. Enzyme production at this temperature was compared with enzyme production at 40°C by the

same organism in the previous experiment.

B. Optimization of Alpha-amylase Production by *B. subtilis* NRRL B3411 in Shake-Flask Culture

1. Nitrogen Source

In order to determine the effects of nitrogen source on enzyme production, ammonium sulfate and rice bran were each substituted for the yeast extract and peptone in the culture medium. The effect of supplementing the ammonium sulfate and the rice bran media with either yeast extract or peptone was also determined. Thus, alpha-amylase production by *B. subtilis* in seven media was compared. Medium 1 is the control medium. The other six media had the following composition (g/1): cassava root powder, 20; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.2, and different nitrogen sources: Medium 2-ammonium sulfate 18; Medium 3-ammonium sulfate, 18 and yeast extract, 5; Medium 4-ammonium sulfate, 18 and peptone, 10; Medium 5-rice bran, 18; Medium 6-rice bran, 18 and yeast extract, 5; Medium 7-rice bran, 18 and peptone, 10.

A second experiment was made comparing enzyme production by *B. subtilis* in a medium containing peptone (10 g/1) solely as the nitrogen source with enzyme production by the same organism in medium 4 and medium 7. In both experiments, the initial pH of the media was adjusted to 7.0. Incubation was made in a rotary shaker at room temperature ($\sim 29^{o}C$) for three days.

2. pH for Enzyme Production

The pH for enzyme production by *B. subtilis* was optimized using medium 4 and medium 7. Enzyme production was initially compared at pH 6.0, 7.0, and 8.0 using medium 4. A second experiment was made using the same medium but during this run, the pH of the medium was adjusted to pH 6.0, 6.25, 6.50, 6.75, 7.00 and 7.25. Using medium 7, the variation of the enzyme production with pH was determined at pH 6.00, 6.50, 7.00, 7.50, 8.00 and 8.50. Concentrated H_3PO_4 (85%) and 3 N NaOH were used to adjust the pH of the media. An enzyme assay was made after three days of incubation in a rotary shaker at room temperature ($\sim 29^{o}C$).

3. Incubation Temperature

Alpha-amylase production by *B. subtilis* was determined after incubation at 30, 35 and $40^{o}C$ in an Eberbach water bath shaker. The medium used for amylase production was medium 7 and the pH was adjusted initially to 7.0. The cultures were assayed for alpha-amylase activity everyday for a period of six days. The final pH of the culture was also determined.

C. Enzyme Assay

1. Determination of Alpha-amylase Activity

The assay procedure used in this study was adopted from the method developed by Fuwa (5). The substrate was a 0.2% starch solution prepared from soluble potato starch.

For the assay, the culture was centrifuged at 8000 rpm for 30 minutes using an IEC centrifuge model HT. The supernatant was diluted with 0.01 M $CaC1_2$ solution. For the *B. subtilis* samples, a dilution of 1:500 to 1:1000 was used while for *B. licheniformis,* the samples for assay were either undiluted or diluted 1:10.

One ml of a 0.5 M acetate buffer, pH 5.7, was added to one ml of the properly diluted sample in a test tube. The volume was made up to 2.5 ml with distilled water. Another tube, which served as the control, contained the same total volume but water was substituted for the enzyme solution. The tubes were kept in a constant temperature water bath at 37°C for about 10 minutes and to each were added 2.5 ml of the starch solution. After an incubation period of 30 minutes, 5 ml of a 1 N solution of acetic acid were added to the tubes. The contents of the tubes were transferred into 250-ml volumetric flasks. Five ml of a mixture of a 0.2% I_2-2% KI solution prepared daily from a stock solution of 1% I_2-10% KI were added to each of the two volumetric flasks and the mixtures were diluted to the mark with water. The absorbance of the samples was determined at 615 nm using an MSE Spectroplus D. The instrument was set to zero against water.

The amylase activity was expressed in terms of the dextrinizing power (D.P.). The latter is defined as the amount of amylase which will produce a 10% fall in the intensity of the blue color of the starch-iodine complex under the conditions of the assay. The dextrinizing power was calculated according to the following equation:

$$D.P. = \frac{A_o - A}{A_o} \times 10$$

where A_o is the absorbance of the control and A is the absorbance of the sample.

2. Protein Determination

The protein content of the samples was determined using the method of Lowry et al. (6). To one ml of the sample were added 5 ml of a solution 50:1 (v/v) of 2.0% Na_2CO_3 in 0.1 N NaOH and 0.5% $CuSO_4.5H_2O$ in 1% sodium or potassium tartrate. After standing for 10 minutes, 0.5 ml of diluted Folin-Ciocalteau phenol reagent (1:1 v/v Folin: H_2O) was added and immediately shaken. The mixture was allowed to stand for 30 minutes. The absorbance of the mixture was read at 720 nm using an MSE Spectroplus D. A standard curve was prepared using crystalline bovine serum albumin (Sigma).

RESULTS AND DISCUSSION

A comparison of alpha-amylase production by *Bacillus subtilis* NRRL B 3411 and *Bacillus amyloliquefaciens* at 29°C is presented in Table 1. The comparative data for enzyme production by the above-mentioned *B. subtilis*

Table 1. Comparison of alpha-amylase production by *B. subtilis* NRRL B3411 and *B. amylo liquefaciens* at 29°C

Incubation Period (days)	Volumetric Activity (D.P./ml)		Specific Activity (D.P./mg protein)	
	B. amylo-liquefaciens	*B. subtilis*	*B. amylo-liquefaciens*	*B. subtilis*
1	750 ± 60	890 ± 40	110 ± 30	178 ± 4
2	1400 ± 600	4320± 70	210 ± 30	1200 ± 300
3	1800 ± 100	8600 ± 100	242 ± 3	1700 ± 70
4	3000 ± 400	7470 ± 0	390 ± 80	1600 ± 300
5	3300 ± 700	8020 ± 0	900 ± 60	1590 ± 40
6	6300 ± 100	8620 ± 80	1600 ± 200	1800 ± 200
7	6930	7450	1707	1192

Experimental Conditions:

Initial cell count

B. subtilis, 1.3 x 10^8 cells/ml

B. amyloliquefaciens, 3.1 x 10^8 cells/ml

Control medium; pH, 7.0; room temperature (~29°C)

strain and *Bacillus licheniformis* NRRL 1001 at 40°C is shown in Table 2. It can be seen from the two tables that *Bacillus subtilis* is the most active producer of alpha-amylase among the three *Bacillus species.* The peak of enzyme production by *B. subtilis* was close to the fifth day of incubation as shown in Table 2. Maximum enzyme production was obtained after five or

Table 2. Comparison of alpha-amylase production by *B. subtilis* NRRL B3411 and *B. licheniformis* NRRL 1001 at 40°C

Incubation Period (days)	Volumetric Activity (D.P./ml)		Specific Activity (D.P/mg. protein)	
	B. subtilis	*B. licheniformis*	*B. subtilis*	*B. licheniformis*
3	1300 ± 400	36 ± 6	310 ± 60	700 ± 400
5	3600 ± 100	83 ± 6	1200 ± 100	32 ± 9
6	2900 ± 500	60 ± 20	880 ± 80	17 ± 6

Experimental Conditions:
Initial cell count
B. subtilis, 1.4 x 10^8 cells/ml
B. licheniformis, 1.2 x 10^9 cells/ml
Medium 7; pH, 7.0; temperature, 40°C

six days of culturing for the three *Bacillus* types. This agrees with published reports of other workers who observed maximal alpha-amylase synthesis after the cultures have reached the stationary phase (7,8,9). In the Ogihara method of industrial alpha-amylase production the enzyme is harvested after five days of culture (10).

The effects of varying the nitrogen source on alpha-amylase production is presented in Table 3. It can be seen that peptone, in combination with ammonium sulfate or rice bran, produced the highest volumetric activities. A separate experiment was conducted in order to determine whether the crucial ingredient in the medium was peptone. The results (C. Valdeavella, unpublished data) showed that there were no significant differences in volu-

Table 3. Effects of nitrogen sources on alpha-amylase production by *B. subtilis* NRRL B3411

Nitrogen Source (g/l)	Volumetric Activity (D.P./ml)	Specific Activity (D.P./mg protein)
yeast extract, 5 peptone, 10	4000 ± 2000	700 ± 400
ammonium sulfate, 18	2 ± 3	4 ± 6
ammonium sulfate, 18 yeast extract, 5	4600 ± 800	2000 ± 700
ammonium sulfate, 18 peptone, 10	8100 ± 400	1650 ± 30
rice bran, 18	930 ± 60	590 ± 70
rice bran, 18 yeast extract, 5	4700 ± 700	1200 ± 200
rice bran, 18 peptone, 10	8000 ± 1000	1400 ± 100

Experimental Conditions:
pH 7.0; room temperature (~ 29°C); enzyme harvested after three days of culture

metric activities of the 3-day old fermentation media containing the following nitrogen sources: (a) peptone, (b) peptone plus ammonium sulfate and (c) peptone plus rice bran. The results indicate that the high yields of the enzyme can be attributed to the presence of peptone in the culture medium. However, recent results (T. Van Den, unpublished data) show that high alpha-amylase activities can also be obtained by using complex nitrogenous ingredients in the medium such as fish meal or soybean meal. Davies (11) gave several reasons for the stimulation of microbial extracellular enzyme production in the presence of complex nitrogenous materials, for example, ready supply of amino acids or peptides and extra supply of growth factors or trace elements.

The pH dependence of alpha-amylase production by *B. subtilis* NRRL 3411 is shown in Table 4. Maximal activities were obtained at pH 7.5, although the variation in pH at different pH values was relatively small and experimental errors were appreciable. A separate experiment was conducted using a different medium on the effect of changing the pH from 6 to 7.25 at incre-

Table 4. Alpha-amylase production by *B. subtilis* NRRL B3411 at different pH values from 6.00 to 8.50

Initial pH	Volumetric Activity (D.P./ml)	Specific Activity (D.P./mg protein)
6.00	800 ± 700	137 ± 5
6.50	1100 ± 300	190 ± 40
7.00	1200 ± 100	190 ± 10
7.50	1500 ± 600	300 ± 100
8.00	800 ± 200	130 ± 90
8.50	800 ± 200	150 ± 30

Experimental conditions:

Initial cell count, 1.5×10^8 cells/ml
Medium 7; room temperature ($\sim 29^{\circ}C$)

ments of 0.25 pH unit. The results (C. Valdeavella, unpublished data) showed that the enzyme activities were practically insensitive to pH variations in the pH range 6.0-7.25. The present findings generally agree with those of Gupta and Sethi (12) who reported an optimal pH range of 6.5-7.5 for alpha-amylase production by *B. subtilis.* It should be pointed out that for the alpha-amylase produced *B. subtilis* NRRL B3411 the previously reported value of the optimal pH for enzyme activity is 6.0 while the pH range of enzyme stability is 5.5-9.5 (13).

The optimal temperature for alpha-amylase production by *Bacillus species* was previously reported by several workers (9, 12, 14) to be in the range 30-40°C. Consequently, for the present work enzyme production was studied only within this temperature range. The results are shown in Tables 5, 6 and 7. Higher enzymatic activities were observed at 30°C compared to the higher temperatures. Unfortunately, different initial cell concentrations were used due to experimental difficulties in determining biomass concentrations. The highest initial cell count, namely 1.8×10^9 cells/ml was used at 30°C while the lowest cell count of 1.4×10^8 cells/ml was used at 40°C. In order to partially correct for the effect of different inoculum sizes, the volumetric activity values were divided by the initial cell concentration to give the activity per cell. The calculated activities per cell were found to be highest at 40°C. However, it should be emphasized that this method of calculation assumes similar growth rates at the three different temperatures. If the growth rate increases with temperature in the range 30-40°C, which is

Table 5. Alpha-amylase production by *B. subtilis* NRRL B3411 at 30°C

Incubation Period (days)	Final pH of Culture Medium	Volumetric Activity (D.P./ml)	Specific Activity (D.P./mg protein)	Activity per cell ($\times 10^6$ D.P./cell)
1	6.25 ± 0.15	100 ± 100	40 ± 40	0.06 ± 0.06
2	6.82 ± 0,02	1800 ± 300	500 ± 100	1.0 ± 0.2
3	6.97 ± 0.02	3500 ± 400	1100 ± 100	2.0 ± 0.2
4	6.98 ± 0.02	6000 ± 800	2270 ± 90	3.3 ± 0.4
5	8.20 ± 0.16	7300 ± 200	2600 ± 400	4.1 ± 0.1
6	8.64 ± 0.04	6800 ± 300	2800 ± 400	3.8 ± 0.1

Experimental Conditions:

Initial cell count, 1.8×10^9 cells/ml
Medium 7; pH, 7.0; temperature, 30°C

Table 6. Alpha-amylase production by *B. subtilis* NRRL B3411 at 35°C

Incubation Period (days)	Final pH of Culture Medium	Volumetric Activity (D.P./ml)	Specific Activity (D.P./mg protein)	Activity per cell ($\times 10^6$ D.P./cell)
1	6.15 ± 0	600 ± 300	200 ± 100	1.1 ± 0.5
2	6.11 ± 0.21	1300 ± 200	350 ± 50	2.5 ± 0.6
3	6.65 ± 0.05	3000 ± 300	700 ± 200	5.6 ± 0.6
4	7.00 ± 0.25	3600 ± 400	1650 ± 60	6.7 ± 0.7
5	8.53 ± 0.07	3900 ± 0	1940 ± 30	7.2 ± 0.0
6	8.83 ± 0.01	4100 ± 100	2000 ± 300	7.5 ± 0.2

Experimental Conditions:

Initial count, 5.4×10^8 cells/ml
Medium 7; pH, 7.0; temperature, 35°C

Table 7. Alpha-amylase production by *B. subtilis* NRRL B3411 at 40°C

Incubation Period (days)	Final pH of Culture Medium	Volumetric Activity (D.P./ml)	Specific Activity (D.P./mg protein)	Activity per cell ($\times 10^6$ D.P./cell)
3	6.16 ± 0.10	1300 ± 400	310 ± 400	9 ± 3
5	8.26 ± 0.05	3600 ± 100	1200 ± 100	26 ± 1
6	8.62 ± 0.12	2900 ± 500	880 ± 80	21 ± 3

Experimental Conditions:

Initial cell count 1.4×10^8 cells/ml
Medium 7; pH, 7.0; temperature, 40°C

expected then the cell concentrations would increase at a faster rate at the higher temperature. This should reduce the value of the activity per cell at the higher temperatures. Thus, the values of the enzyme activity per cell at 40°C based on the actual, *not* initial cell concentration are expected to be much less than those listed in Table 7. Needless to say, an actual experiment using exactly the same initial cell concentrations at different temperatures need to be done in order to resolve the issue.

In summary, the present experimental results show that there are good prospects for using *Bacillus subtilis* NRRL B3411 as producer of alpha-amylase. High enzyme activities were obtained after three days of batch culture at 29°C. Compared to *Bacillus licheniformis* NRRL 1001 and a local isolate of *Bacillus* amyloliquefaciens, this *Bacillus* strain needs to be studied further using a suitable fermenter in order to optimize alpha-amylase production in a larger scale.

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