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Alkaline α-amylaseProduction from Bacillus megaterium

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Abstract

The enzyme expressed from strain L-49 was 2.01 times higher than that of original strain. Strain L-49 can grow on culture plate with 50μg/mL ampicillin. The synthesis of α-amylase was greatly suppressed when strain L-49 was grown on monosaccharide such as glucose and polysaccharide at the same time cell concentration was low. Amylase production was enhanced when the bacterium was grown on starch and dextrin. Among different nitrogen sources tried, yeast extract was found to be the best followed by panpeptone, peptone, meat extract, bean meal, and corn steep liquor. The average rate of enzyme production was enhanced for 3~4 times in fermentation time from 24h to 44h.. The sugar uptake rate has also increased. Low oxygen supply rate enhanced the rate of strain propagation but depressed the enzyme production. Hence it is benefit to obtain high enzyme activity that agitation speed maintained not lower than 400r/min and aeration rate maintained greater than 1:1vvm.

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I. INTRODUCTION

Alkaline alpha-amylases, belong to these enzymes, are used not only in wash industries but also in textile and paper industries as well as food production. In addition, hydrolyzing starch in alkaline conditions is benefit to gelatinization at low temperature. Therefore, study of alkaline alpha-amylase may provide a new method of hydrolyzing starch. Alpha-amylase abtained from *Aspergillus niger* is acid-resistant. These amylases all have been produced in industry. In late eighties, strains which produced highly thermostable alkaline alpha-amylase were isolated from different soil samples by India scholar[1-4]. Alkaline alpha-amylase has not been solded as commodity yet. Previously Jar et al[5]. isolated a bacterial strain, *Bacillus megaterium* L-49 has been isolated and identified that produces alkaline α-amylase. The cell is ellipsoidal, about 1.0-1.2 × 3.0-3.6μm in diameter, Gram-positive, motile, and central partial central. Growth occurs in media containing 7% of NaCl. This strain could utilize D-glucose, lactose, xylose, sucrose, mannose, and maltose, and but it does not utilize D-fructose, and glycogen. Among the various concentrations of saturated ammonium sulfate, the retractation ratio in range of 70 to 100% was about 93%. However, in the case of acetone, it was about 98.7%. EDTA has activating effect and Ca^{2+} has no effect on alkaline α-amylase activity. The alkaline α-amylase has low thermal stability. The optimal temperature for reaction is 50℃. The alkaline α-amylase activity maintained stabilizing at pH 6-11 and the optimal pH for reaction was 9-10.

In this article, we deal with a alkaline-amylase producing strain of Bacillus elaterium which secretes α -amylase in high alkaline media(pH11). We obtained a kind of strain L-49 which has comparative large ability of producing alkaline α-amylase by mutagenesis using alkaline alpha-amylase producing strain 9-A2 screening from the soil samples as original strain. This includes the isolation and identification of the organism, cultural conditions, fermentation technics in 14-L fermenter and properties of the enzyme.

II. METERIALS AND METHODS

1. Strain and Cultural Conditions

Enzyme production was carried out by growing the organism in a liquid medium with the same composition as the isolation medium. Fifty milliliters medium was placed in a 500-mL conical flask and inoculated with the culture. Fermentation was continued at 30℃ for 72h in a reciprocating shaker. Before assay, the cells were separated by centrifugation at 5000g. The clear supernatant was used as crude enzyme preparation. The pattern of enzyme production and growth was also studied in a 14-L self-control fermenter (MJ-N14LL.E.MARUBISHI, Co.LTD) with optimum fermentation conditions. The aeration rate maintained at 1:1-1:1.25vvm, the agitation speed was kept constant at 400r/min, 30℃,

inoculation amount 6-10 %(v/v), and 10-L liquid medium. A vegetable oil was used to check for foam formation. Strains were isolated from different soil samples using a selective medium, which contains 0.5% yeast extract, 0.5% peptone, 1%soluble starch, 0.1%k_2HPO_4, 0.02%$MgSO_4 \cdot 7H_2O$, 1%Na_2CO_3,.For the solid medium, agar was used at a concentration of 2.5%.

2. Amylase activity assay

0.2ml of Methyl ethane sulphonate was added into 20ml of diluted culture solution, and incubated at 30℃ for 40 and 60min. After detoxifcation carried out by dilution, the organism was cultured for 16h and subsequently diluted as unicellular suspended liquid by saline. This solution was irradiated by ultraviolet radiation (15W,30cm), and then 0.1ml of this solution was added on the culture plate containing 50μg/ml ampicillin and cultured at 30℃ for 3 days for selecting the mutant[4]. The strain was kept at 4℃ on the agar slants containing 0.5%yeast extract, 0.5%peptone, 0.1%k_2HPO_4, 0.02%$MgSO_4 \cdot 7H_2O$, 1%Na_2CO_3 and 2.5%agar.

3. Analytical Procedure

Extracellular amylase activity was determined by measuring the decrease in iodine color. The reaction contained 1mL diluted enzyme (cell from supernatant), 3mL boracic acid-borax buffer (pH9.0) and 1mL of 11% starch solution incubated at 40℃ for 10min. The reaction was stopped by adding 0.5mL of this solution to 1mL of 1N HCl. Two milliliters of 0.01% iodine solution and 6.5mL distilled water were added to this acidified solution. The optical density of blue-colored solution was determined at 680nm. The same procedure was repeated using 1mL distilled water instead of the enzyme sample in order to measure the optical density without the enzyme. *One unit of enzyme activity(DUN)* is defined as the quantity of enzyme that causes 10%reduction of blue color intensity of starch-iodine solution at 40℃ in 10min. The starch concentration was determined spectrophotometrically by using iodine solution. Growth was estimated in terms of dry weight of cell. Iodine color decreasing rate measurement: 5ml of 4% starch was added to the enzyme solution at 60℃. The variety of iodine color and viscosity was determined at different reaction time.

III. RESULTS AND DISCUSSION

1. Mutagenesis of strain

Alkali-tolerant *Bacillus megaterium* 9-A2 was mutagenized by ultraviolet irradiation and ethereal sulfate alternately. Several strains that yielded enzyme steadily was obtained shown in Table 1. The enzyme expressed from strain L-49 was 2.01 times higher than that of original strain. Strain L-49 can grow on culture plate with 50μg/mL ampicillin.

Table 1. Mutagenesis of strain for High yield mutant

Strain	Enzyme activity (DUN/ml)	Strain	Enzyme activity (DUN/ml)
9-A2	298	L-29	573
B-29	430	L-39	589
12	532	L-53	571
G3	560	L-58	566
L-49	598		

2. Production of Enzyme in Erlenmeyer Flask

The effect of carbon source, nitrogen source, salt concentration, gas through-puts, time fermentation and temperature on enzyme production was observed in a 500mL Erlenmeyer flask. The initial pH was 8~10,inoculation amount 5%,and seed age 18~24h. The effect of different carbon sources, such as dextrin, glucose, sucrose, maltose, lactose, galactose, xylose, fructose and soluble starch, on α-amylase production was studied. The synthesis of α-amylase was greatly suppressed when strain L-49 was grown on monosaccharide such as glucose and polysaccharide at the same time cell concentration was low. Amylase production was enhanced when the bacterium was grown on starch and dextrin. However, the strain growth with these substrates was also enhanced. These observations illustrate that growth rate and α-amylase productions were in direct proportion at a certain extent. Using lactose as carbon source was benefit to strain growth. (Table 2).

Table 2. Effect of carbon sources on α-amylase production

Carbon source (%)	Final pH	Cell concentration OD_{680}	Enzyme activity(DUN/mL)
Dextrin (2)	9.0	0.578	742
Glucose (2)	8.5	0.426	125
Sucrose (2)	9.0	0.377	141
Maltose (2)	8.5	0.324	128
Lactose (2)	8.5	0.625	163
Galactose (2)	9.2	0.339	320
Xylose (2)	9.0	0.394	178
Frutose (2)	9.0	0.121	67
Soluble starch (2)	9.0	0.541	738

Various complex nitrogen sources were added separately to the medium to assess their effect on enzyme production. Among different nitrogen sources tried, yeast extract was found to be the best followed by panpeptone, peptone, meat extract, bean meal, and corn steep liquor (Table 3).

Table 3. Effect of organic nitrogen on α-amylase production

Organic nitrogen source (%)	Final pH	Enzyme activity (DUN/mL)
Panpeptone (2)	8.5	325
Peptone (2)	8.8	383
Yeast extract (2)	9	601
Meat extract (2)	8.5	326
Bean meal (2)	9	416
Corn steep liquor (5)	8.5	432
Yeast extract (1) and peptone (1)	8.5	594

Effect of C:N on enzyme production was carried out by growing the organism in a liquid medium placed in Erlenmeyer Flask. Starch, yeast extract and peptone were added in the medium at various concentrations. The result was that effect of enzyme production was best when proportion of carbon and nitrogen sources was 2:1 (Table 4)

Table 4. Effect of C:N on α-amylase production

Starch (%)	Yeast extract(%)	Peptone (%)	C:N	Final pH	Enzyme activity (DUN/mL)
2.5	0.5	0.5	2.5:1	8.8	534
2	0.5	0.5	2:1	9	659
8	2	2	2:1	9.2	206
2	1	0	2:1	8.8	677
2	1	1	1:1	9	477
1	0.5	0.5	1:1	9	457
4	2	2	1:1	9	410
2	1.5	1.5	1:1.5	8.7	561
2	2	2	1:2	8.4	365

Liquid medium was added to several 500-mL Erlenmeyer flasks in various quantities and inoculated with the culture. Fermentation was continued at 30℃ for 48h. Enhancement of Oxygen-dissolved rate was benefit to high enzyme

production (Figure 1).

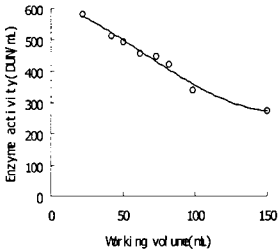

Figure 1. Effect of gas through-puts on α- amylase production.

Liquid medium was inoculated with the culture. Fermentation was continued at 30℃ for various hours to observe the optimum culture time. That is 48h (Figure 2).

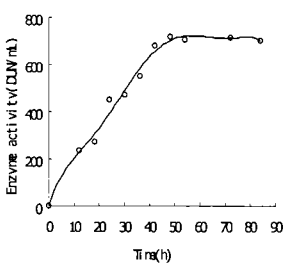

Figure 2. Effect of culture time on α -amylase production.

3. Production of Enzyme in Fermenter

Figure 3 shows the production of the enzyme in a 14L fermenter with optimized conditions. The aeration rate maintained at 1:1 and 1:1. 25vvm, the agitation speed was kept at 200r/min and 400r/min. Enhancement of oxygen-transfer rate increased the rate of enzyme production. The average rate of enzyme production was enhanced for3~4 times(The second and third batch contrast to the first batch) in fermentation time from 24h to 44h.. The sugar uptake rate has also increased. Low oxygen supply rate (The first batch) enhanced the rate of strain propagation but depressed the enzyme production. In addition, increasing inoculation amount (the second batch contrast to the third batch) attributed to shorten fermentation periods. We could observe from the oxygen-dissolve curve that dissolved oxygen concentration decreased to 5% when the fermentation continued to 30h with the conditions of aeration rate 1:1vvm and agitation speed 200r/min in the first batch. Subsequently, strain growth rate was accelerated(Curve 1of figure 3) when the fermentation continued to 30~40h while enzyme activity changed a little(Curve 1of figure 3). Effect of the second and third batch attained the goal, hence it is benefit to obtain high enzyme activity that agitation speed maintained not lower than 400r/min and aeration rate maintained greater than 1:1vvm.

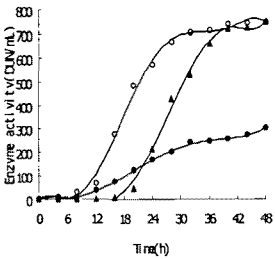

Figure 3. Relation between fermentation and rate of oxygen transfer

1. The first batch, aeration rate 1 : 1.0, inoculation amount6%, 200r/min, 30h later 300r/min

2. The second batch, aeration rate 1 : 1.25, inoculation amount 6%, 400r/min

3. The third batch , aeration rate 1 : 1.25, inoculation amount 9%, 400r/min

IV. CONCLUSION

The results presented in this article show that enzyme activity could attain 740DUN/mL in 14-L fermenter at the conditions of agitation speed 400r/min, aeration rate 1:1.25vvm and fermentation period 44h. Strain L-49 can secrete α -amylase in high alkaline media. It's characteristic of this kind decrease the chance of contamination in fermentation. The enzyme activity indicated that this enzyme can be used in alkaline scour and was resistant to high calcic water.

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