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Production and Preservation of α-Amylase from *Bacillus* sp. Y-124

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Bacillus sp. Y-124로부터 α-Amylase의 생산 및 그 보존성에 관한 연구

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Abstract

Microorganisms from the waste water of starch industry, were isolated and a strain, Y-124, possessing a powerful enzymic activity was selected and identified as a member of the genus *Bacillus*. The ideal cultural condition for the formation of α-amylase form *Bacillus* Y-124 and its preservation was investigated in connection with the biotechnological and industrial approach to the bulk enzyme production. High yield of α-amylase was observed in medium containing casein as well as calcium pantothenate in this work. Calcium ions were found to have an effect in forming this particular enzyme. Ammonium phosphate dibasic was an important inorganic nitrogen source for the formation of α-amylase. And preservation of this enzyme was greatly affected by calcium or sodium salts. The addition of calcium carbonate or sodium sulfate presented the most effective result for the prevention of its denaturation to various factors. The above data was obtained with crude enzyme preparation.

Introduction

The earliest known extracellular enzyme was α-amylase, used in large quantities for industrial purposes, especially in starch industries. Therefore, numerous publications to the catalytic activities of α-amylase were devoted to its study; not only for its academic interest but also for its applications to many fields of the related industries[1~8] Most of reports dealt with the isolation and crystallization from the origin of molds and bacteria[9,10]. α-Amylases have been found in animals, plants, molds, and bacteria. The molecular weight of all α-amylases are about 50,000 containing one atom of calcium per molecules. However they show differences in the compositions of their constituent amino acids in their cofactors and in heat stability. Thermostable α-amylase now attracts scientific interest from a biological point of view in relation to the adaptation

mechanism of living organisms to extreme conditions under which ordinary heat-sensitive cellular constituents are destroyed. The thermophilic bacteria were first described by Miquel in 1888.

In view of α-amylase produced by the genus *Bacillus*, some studies have been conducted so far. Coleman and Grant observed that glucose and glycerol did not cause the catabolic repression on amylase synthesis in *Bacillus amyloliquefaciens* when they were added to growing culture of this organism[11]. But Meers, Yamamoto and Saito reported that glucose or other low molecular weight metabolizable sugars repressed α-amylase formation[12]. Anustrup concluded that α-amylase synthesis in *Bacillus licheniformis* is less calcium dependant than in *Bacillus subtilis*[9]. After the crystallization of *Bacillus subtilis* α-amylase was first accomplished by Meyer, Fuld, and Bernfeld in 1947. *Bacillus subtilis* α-amylase was extracted from a commercial concentrate in powder form. Manning and Campbell studied *Bacillus stearothermophilus* α-amylase and demonstrated its isolation in crystalline form. And Campbell and his co-workers, had crystallized a heat-stable α-amylase from *Bacillus stearothermophilus* strain 503-4 in 1961[6]. It was reported in 1970 by Isono that *Bacillus stearothermophilus* produced an α-amylase having greater heat stability that the α-amylase produced by the same organism, but their electrophoretic mobilities and optimal temperature appeared to be the same indicating the difference in amino acid[1]. And there are several studies on some general properties of this enzyme [13,14] or on the mechanisms of mutation with regard to its hyperproduction[15,16].

Partial purification of the amylase from *Bacillus polymyxa* was achieved by the use of ammonium sulfate and organic solvent precipitations. The investigation of the action pattern was qualitatively and quantitatively exerted by paper chromatography describing the change in optical rotation[17]. *Bacillus subtilis* α-amylase is a calcium metalloenzyme normally found in the form of a dimer, in which the two protein moieties are linked by an atom of zinc. In the amino acid composition of α-amylases from *Bacillus subtilis*, the absence of sulfhydryl groups and of disulfide linkage were confirmed for cysteic acid in hydrolysates, indicating that the *Bacillus subtilis* α-amylase molecules were composed of 406 amino acid residues[18].

In the presence of zinc, a dimer is formed which contains one atom of zinc which is thought to establish cross link between two monomeric molecules[13]

Some physico-chemical and biochemical properties of a crystalline α-amylase from *Bacillus stearothermophilus* have been investigated. It was shown that thermophilic α-amylase was more resistant to higher temperature than *Bacillus subtilis* α-amylase. The molecular weight (48,000), the intrinsic viscosity (0.032 dl/g), and the optical rotatory dispersion were measured. A difference in the thermophilic α-amylase from *Bacillus subtilis* α-amylase was found in the circular dichroism spectra in the 250-320 nm region and in the amino acid composition[19].

The increased production of extracellular thermostable α-amylase by *Bacillus licheniformis* was obtained by using vitamins, amino acids, and nitrogen sources. Of the carbohydrates examined, galactose was the best carbon source. And metal ions such as Na^+, Cu^{2+}, Cd^{2+}, Ag^+, and surfactants, including Tween-80 and Triton X-100 were discussed on the formation of α-amylase[9].

In contrast with above investigations, an amylase inhibitor possessing an inhibitory activity on α-amylase was also obtained from a subspecies of *Streptomyces diastaticus*[14].

This communication dealt with the ideal cultural conditions fol the formation and preservation of α-amylase from Y-124 identified as a variety of the genus *Bacillus* in terms of substrate and food additives.

Materials and Methods

1. Microorganism

Organisms used in the experiment were initially isolated from the waste water of starch industry and a strain, Y-124, possessing a powerful enzymic activity was selected from batch cultures and

identified as a member of the genus *Bacillus*[20,21]. *Bacillus* Y-124 was used throughout the present experiment.

2. Cultivation

The basal medium was composed of 5% soybean, 1.5% corn starch, 1.2% $(NH_4)_2HPO_4$, 0.01% KCl, and 0.01% $MgSO_4 \cdot 7H_2O$ (pH 7.0). The selected strain was cultivated in the above media at 37°C for 34 hrs or 72 hrs on the reciprocal shaker (120 r.p.m. at 6 cm stroke).

3. Preparation of enzyme solution

The crude enzyme was used throughout the experiment. 150 ml of medium was placed in a 500 ml shaking flask, sterilized and inoculated with a loopful of an actively growing slant culture. The cultivation was conducted at 37 C on the reciprocal shaker. The resulting medium was centrifuged at 3,000 r.p.m. for 20 minutes. Then 0.01 M acetate buffer (pH 6.0) and 0.02 M $CaCl_2$ were added to the supernatant adjusted to pH 6.0. The mixture was dialyzed in cellulose tubing.

4. Assay procedures of α-amylase

The modified Blue Value method, showing changes in the iodine color of the assay mixture, was employed. The amount of decrease in the absorbance of iodine was a measure of the activity of α-amylase. 1 ml of suitably diluted enzyme solution was incubated with 10 ml of 1% potato starch solution adjusted to pH 6.0 with an acetate buffer (pH 6.0) and heated in water bath as substrate for 10 min. Then 1 ml of the reaction mixture was pipetted into a 50 ml flask containing 10 ml of 0.1 M HCl. After the addition of 10 ml of 0.005% I_2-0.05% KI solution to 0.5 ml of that solution, the flask was heated for 2 min, -log transmittance of the resulting colorized solution was measured at 660 nm. One DP (dextrinizing power) is the amount of enzyme catalyzing a 1% decrease in 10 ml of blue value of 1% starch solution per minute at 40 C.

$$DP=\frac{D-D'}{D}\times\frac{100}{10}\times N$$

D: -log transmittance of the diluted enzyme solution

D': -log transmittance of blank

N: Dilution time of enzyme solution

Results

Generally, the cultural conditions such as composition of medium, metal ions, inorganic salts and amino acid have been known to be significant in the production of secondary metabolites. Therefore, it is important to investigate the formation of α-amylase or other phenomena from this strain. All chemicals used through this work were products of the certified reagent grade.

Effect of calcium ion and other substance on the formation of α-amylase

α-Amylase activity from *Bacillus* Y-124 cultivated in the basal medium or the starch-free basal medium for 72 hrs was tested and shown as in Table 1. Calcium salts enhancing the formation of α-amylase of this strain were calcium carbonate and calcium phosphate dibasic in both media, but calcium hypochlorite remarkably repressed an α-amylase formation. It was noticed that casein was an inducer for the maximal formation of this enzyme and calcium pantothenate and D-ribose were also important

Table 1. Effect of Calcium Ion and Other Substance on the Formation of α-Amylase.

Chemical (0.2%)	Relative activity	
	Basal medium	Starch-free basal medium
Calcium carbonate	90	70
Calcium phosphate nonobasic	60	60
Calcium phosphate dibasic	95	95
Calcium nitrate	60	55
Calcium hypochlorite	35	45
Calcium pantothenate	95	92
Dextrin	84	92
Potassium chloride	57	57
2,4-D	45	40
Casein	100	97
D-ribose	94	92
Dextrin Magnesium chloride	90	90

stimulators for α-amylase production in both media; however, potassium chloride and 2,4-D were repressors for α-amylase formation. And in general, the activity of α-amylase from this strain cultivated in starch-containing media was higher than that in starch-free media.

Effect of nitrogen sources on the formation of α-amylase

The effect of nitrogen sources on the formation of α-amylase was determined after an incubation period of 36 hrs. It was observed that ammonium oxalate and urea caused more poor enzyme-formation than control indicating that these chemicals had an inhibitory or a repressive action against α-amylase activity or synthesis. However, ammonium phosphate dibasic, ammonium sulfate, and ammonium nitrate were inclined to enhance the enzyme production. The order of nitrogen correlated with increasing enzyme formation was ammonium phosphate dibasic, ammonium sulfate, ammonium nitrate, and their respective concentration optima were 0.1, 0.01, and 0.01% (Table 2). This result indicated that ammonium phosphate dibasic played an important role in the formation of α-amylase during fermentation. But other nitrogens, such as potassium nitrate and sodium nitrate, had little or no effect on the enzyme production.

Effect of mixture of soybean-starch medium and some chemicals

Fermentation of culture broth was carried out for 36 hrs. In the mixture of ammonium phosphate dibasic and ammonium sulfate, *Bacillus* Y-124 showed a considerable amount of enzyme production. In general, a mixture of chemicals enhanced the enzyme accumulation in comparison to the addition of a single chemical. The basal medium used in this experiment was composed of 5% soybean and 1.5% starch, and the chemicals were 0.1% ammonium phosphate dibasic, 0.01% ammonium sulfate, 0.01% ammonium nitrate, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.001% KNO_3 (Table 3).

Table 2. Effect of Nitrogen sources on the Formation of α-Amylase.

Nitrogen source	Dextrinizing power(units/ml)			
	1%	0.1%	0.01%	0.001%
Ammonium nitrate	346	356	400	320
Ammonium sulfate	340	376	425	330
Ammonium phosphate dibasic	402	440	410	326
Ammonium oxalate	107	185	202	205
Urea	130	215	288	295
Sodium nitrate	340	330	368	340
Potassium nitrate	336	320	346	350
None	340			

Table 3. Effect of Mixture of Soybean-Starch Medium and Some Chemicals on the Formation of α-Amylase.

Mixture of chemical	Dextrinizing power (units/ml)
Ammonium sulfate	420
Ammonium nitrate	400
Magnesium sulfate	385
Potassium nitrate	350
Ammonium phosphate dibasic +Ammonium sulfate	495
Ammonium phosphate dibasic +Ammonium nitrate	480
Ammonium phosphate dibasic +Magnesium sulfate	450
Ammonium phosphate dibasic +Potassium nitrate	475
Ammonium sulfate +Ammonium nitrate	430
Ammonium sulfate +Potassium nitrate	410
Ammonium sulfate +Magnesium sulfate	440
None	340

Effect of cultural history on the formation of α-amylase

As the first culture, a medium containing 5% soybean and 1.5% starch with 0.1% of each chemical was prepared in a 10 ml test tube and sterilized, inoculated, and cultivated on the reciprocal shaker for 24 hrs. After being cultivated for the first culture, this culture liquor for the second culture was transferred aseptically to a 300 ml shaking flask containing the basal medium and cultivated actively on the shaker for 48 hrs and the enzymic activity was measured. The results from the first-second culture are presented in Table 4. The

Table 4. Effect of Cultural History on the Formation of α-Amylase.

First culture	Second culture
Addition (0.1%)	Dextrinizing power(units/ml)
Citrate	795
Glucose	745
Glutamate	770
Inositol	720
Levulose	630
Mannose	700
Tryptophan	650
Magnesium sulfate	660
None	630

The iiolated strain Y-124 was cultivated in test tube containing 5% soybean and 1.5% starch with 0.1% of each chemical for the first culture. After being cultivated, the cultivated test tubes were transferred to the basal medium-containing flask and cultivated for the second culture. The resulting enzymic activity was measured.

results indicated that enzyme production showed the maximum accumulation in cases orderly citrate, glutamate, glucose, and inositol, but in other cases, there was only a small amount of accumulation.

Effect of calcium ions on the preservation of α-amylase

To study the preservation of α-amylase produced from this strain, 0.5% calcium salts were added to the enzyme solution adjusted to 720 DP with 0.01 M acetate buffer, and an antiseptic agent was added. The remaining activity was measured after incubation for various intervals. The results are shown in Table 5. *Bacillus* Y-124 α-amylase was noticeably inactivated after 48 hrs with calcium hydroxide treatment, but other calcium salts made enzyme activity stable. The most effective calcium salt for enzyme stability in long-term preservation (2 months) was calcium carbonate, the second most effective salts were orderly calcium sulfate and calcium phosphate monobasic. Thus these three calcium salts were significant for enzyme stability in this particular strain.

Table 5. Effect of Calcium Ions on the Preservation of α-Amylase.

Chemical (0.5%)	Dextrinizing power (units /ml)		
	48 hrs	1 month	2 month
Calcium carbonate	716	694	670
Calcium chloride'	712	670	610
Calcium hypochlorite	410	325	200
Calcium hydroxide	490	440	250
Calcium nitrate	710	650	615
Calcium sulfate	710	670	645
Calcium phosphate monobasic	710	656	630
Calcium phosphate dibasic	705	625	595

The starting dextrinzing power: 720 units/ml

Effect of sodium ions on the preservation of α-amylase

To study the effect of sodium ions on the preservation of α-amylase produced by *Bacillus* Y-124, an antiseptic agent was initially added to the enzyme solution adjusted to 720 DP with 0.01 M acetate buffer (pH 6.0). After the enzyme solution containing various sodium salts (0.5%) were incubated for 48 hrs, for 1 month or 2 months at 37 C respectively. The dextrinizing

Table 6. Effect of Sodium Ions on the Preservation of α-Amylase.

	Dextrinizing power (units/ml)		
Chemical (0.5%)	48 hrs	1 month	2 month
Sodium acetate	710	650	615
Sodium borate	713	635	590
Sodium carbonate	714	655	630
Sodium chloride	702	617	564
Sodium citrate	705	620	570
Sodium molybdate	700	575	515
Sodium sulfate	710	675	650
Sodium thiosulfate	717	645	600
Sodium phosphate monobasic	705	600	540
Sodium phosphate dibasic	707	600	525
Potassium sodium tartrate	690	576	520

The starting dextrinizing power: 720 units/ml

power of the resulting enzyme was determined. The results are shown in Table 6. Enzyme solution containing various sodium salts did not show the denaturation of the enzyme as a result. After being incubated for 48 hrs, the sodium salts employed presented a slight loss of enzymic activity. But after 1 month, in case of sodium acetate, sodium carbonate, sodium borate, sodium citrate, sodium sulfate and sodium thiosulfate, the resulting enzyme was more or less stable; however, the incubation of the enzyme solutions containing sodium molybdate, sodium phosphate monobasic or dibasic, and potassium sodium tartrate gave arise to a reduction in the dextrinizing power. After being incubated for 2 months, which was relatively long-term, the enzyme solution added sodium ions was stable in the orders of sodium sulfate, sodium carbonate, sodium acetate, sodium thiosulfate, and sodium borate. But sodium molybdate, sodium phosphate monobasic or dibasic, and potassium sodium tartrate showed unstable results.

Discussion

Our communications dealt with recipes for medium suited to the formation of α-amylase by the genus *Bacillus*, Y-124 and its preservation for the industrial purposes. From the cultural data, it was obvious that this strain possessed several similarities with other reports.

Some substances as surfactants or phosphatidylinositol was claimed to have a general beneficial effect on enzyme production. Our experiments showed that α-amylase fermentation could be effectively carried out with simple media containing a source of proteins, carbohydrates and some nutrient salts. It was apparent that casein had an accelerating effect or enzyme production as complex nitrogen source and appeared to be best for high yield of α-amylase. Calcium salts tested were found to possess a stimulatory or repressive action in forming α-amylase. This finding was generally agreed and shared by many authors that all α-amylases experimented contained at least one gram atom of firmly bound calcium per mole of enzyme[11,22,23,24,25]. In contrast, the results also revealed that one of features was different from the above general agreement. Both calcium hypochlorite and calcium nitrate as calcium salts caused the strong repression in forming α-amylase with regard to their valency. Regarding the effect of organic or inorganic nitrogen sources on the formation of α-amylase, it was observed that ammonium sulfate, ammonium chloride, ammonium nitrate, sodium nitrate and thiourea caused both poor growth and enzyme production[9]. However, it was apparent from our experiment that on the other hand, ammonium sulfate and ammonium nitrate showed relatively good growth and enzyme formation whereas ammonium oxalate and especially urea caused a slight repressive effect on the production of α-amylase. From these results, it could be concluded that a certain concentration of ammonium salts incorporated in medium resulted in highly significant increase in cell growth and enzyme production. And it was shown that phosphate caused a stimulatory effect on α-amylase production. Chandra *et al.* reported in their studies of thermostable α-amylase by *Bacillus licheniformis* CUMC-305 that ammonium phosphate dibasic was the best phosphate source for α-amylase production as compared to potassium phosphate monobasic or dibasic[9]. This result was in an agreement with our examination that ammonium phosphate enhanced the maximum enzyme production among inorganic chemicals used at the concentration of 0.1 per cent. Enzymes produced by microorganisms become degraded and autolyzed. Therefore, the stability of liquid products often presents a problem. There was a report that the enzyme lost about 10 per cent of its activity by standing for 96 hrs. at 0°C and about 15 per cent at 40°C. The crude enzyme preparation, therefore, was found to be relatively unstable in solution[17].

Our communication provided the prolonged preservation of the enzyme preparation incorporated calcium or sodium salts. It was found that enzyme preparation incorporated calcium

carbonate lost about 7 per cent of the starting activity when incubated for 2 months at 37 C.

Instead, calcium hypochlorite showed about 72.2 per cent of loss in the starting activity and in case of calcium hydroxide, there was about 65.2 per cent of loss in the activity. And an enzyme preparation incorporated sodium salts presented comparatively a small amount of loss in the starting activity. Sodium sulfate, sodium carbonate, and sodium acetate lost about 9.7, 12.5, 14.5 per cent in the starting activity, respectively. From these result, it was assumed that calcium or sodium ions played a significant role in preventing from the degradation of α-amylase. These interpretations confirmed the suggestions of others[26,27,28,29,30] and roughly coincided with that calcium protected the thermal denaturation and proteolytic degradation of this particular enzyme.

More detailed investigations of the enzyme and identification of the strain Y-124 will be continued and presented elsewhere.

요 약

산업폐수로부터 강력한 α-amylase 생성능력을 가진 미생물을 분리하여 검토한 결과 *Bacillus* 속 (Bacillus Y-124)으로 판명되었으며, *Bacillus* Y-124로부터 α-amylase의 생산성을 최적화하기 위해 배양조건 및 생성된 효소의 안정성을 유지하기 위한 제반 영향들을 검토하였다.

기본 배지내의 casein 및 Ca-pantothenate의 첨가는 본 효소의 생산에 좋은 인자로 작용하였으며 calcium이온 역시 효소합성에 관여하는 것으로 나타났다. 또한 $(NH_4)_2HPO_4$는 본 미생물의 효소생성에 중요한 유기질소원으로 작용하였다.

생성된 본 효소의 보존에 대한 안정성의 유지에는 calcium 및 sodium염의 영향이 컸으며 특히 $CaCO_3$ 및 Na_2SO_4의 첨가는 이 효소의 변성요인을 제거하는데 효과적인 보존제의 하나로 인식되었다.

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