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EFFECT OF CARBON SOURCES ON THE PRODUCTION OF ALKALINE PROTEASE BY *ASPERGILLUS ORYZAE*

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The effect of 31 different non-nitrogenous as well as nitrogenous carbon sources on the biosynthesis of alkaline protease by *Aspergillus oryzae* IMI-17299 was examined using amonium sulphate as a nitrogen source. Among them mono, di and polysaccharides tried, fructose, maltose and maize starch gave the highest level of the enzyme formation i.e., 13.4, 12.6 and 14.5 units/ml respectively. Arabinose, mannose, sorbose, xylose, mannitol, dextrin, inulin, glycerol, ethyl alcohol, methyl alcohol, paraffine, sodium acetate and lactic acid were poor producer of the enzyme. Molasses, both cane and beet, were the best among the non-nitrogenous carbon sources giving the enzyme yield of 16.4 and 15.2 units/ml respectively. Out of the nitrogenous carbon sources ground nut flour, soybean flour and wheat bran gave better yield of the enzyme, i.e., 12.2, 15.2 and 13.2 units/ml respectively. The addition of various oils to the medium stimulated the enzyme production to different extent. Ground nut, corn and rice bran oils showed greater stimulatory effect, soybean, sunflower, castor and coconut oils exhibited very little or no stimulatory effect. The addition of chlorides of sodium, potassium and calcium to the medium increased the yield of the enzyme; the other salts did not enhance the enzyme formation to any appreciable extent.

Key words: Carbon sources, Alkaline protease, *Aspergillus oryzae.*

Introduction

Alkaline proteases find applications in bread making, brewing and leather industries. Various workers have studied the effect of carbon sources on the production of proteases by *Aspergillus oryzae, Aspergillus niger* and bacteria using monosaccharides, disaccharides, polysaccharides and industrial by-products as carbon source [1-5]. In our earlier paper we reported the effect of various nitrogen sources on the production of alkaline protease by the strain of *Aspergillus oryzae* IMI- 17299 [6]. It was observed that inorganic nitrogenous compound, ammonium sulphate, a locally produced cheap fertilizer is an economical nitrogen source for the production of the enzyme.

The present study has been undertaken with a view to (i) selecting a cheap carbon source (especially agricultural by- products) such as molasses, oil cakes etc. which are abundently available in the country, are not being properly utilized, and (ii) formulating a new medium for the propagation of *A. Oryzae* to get comparatively better yield of the enzyme in the shake culture. To achieve these goals glucose in the original medium has been resplaced by various nitrogenous as well as non-nitrogenous carbon compounds. Effect of various vegetable oils and inorganic salts on the yield of the enzyme has also been investigated.

Materials and Methods

Organism. The fungus used in the present investigation was *Aspergillus oryzae* IMI-17299 which has been reported to give a high yield of alkaline protease [6].

Stock culture. The stock cultures were maintained on Czapeck Dox agar medium which contained (gm/l) sucrose 30.0, $NaNO_3$ 2.0, KH_2PO_4 0.50, KCl 0.50. $FeSO_4$-$7H_2O$ 0.01, agar 25.0 (pH of the medium 7). Cultures were grown at 30° for 5-7 days and then stored in a refrigerator.

Fermentation medium. The organism was grown on Nakagawa medium [7] modified by these authors [6] to see the effect of nitrogen source containing the following ingredients:-

(gm/l) glucose 30, K_2HPO_4 1, KH_2PO_4 0.5, $MgSO_4$-$7H_2O$ 0.5, $FeSO_4$- $7H_2O$ 0.01, $ZnSO_4$-$7H_2O$ 0.01, $(NH_4)_2SO_4$ 6.6, $CaCO_3$ 10. The components of the medium were dissolved in distilled water with the exception of $CaCO_3$ which was separately suspended in water, sterilized and mixed accepticaly with the other sterilized portion of the medium, (pH 7).

For studying the effect of non-nitrogenous carbon sources on the biosynthesis of the protease, various monosaccharides, disaccharides, polysaccharides and alcohols were added to the original medium with amonium sulphate as sole source of nitrogen [6]. The carbon sources were added to the original medium equivalent to 3% glucose (on carbon basis) as a sole source of carbon in place of glucose. Polysaccharides and industrial by- products were added in 3% w/v basis.

Shake flask culture. Spores of the cultures grown on Czpaeck Dox agar medium were suspended in water and counted. One ml of the inoculum containing 10^6/ml spores was transferred to each of the three sterilized 500 ml flasks, containing 24 ml fermentation medium. The flasks were incubated at 30° on a rotary shaker for 72 hrs. The culture

filtrate was assayed for protease activity.

Vegetable oils. Most of the vegetable oils were purchased from the local market, except ground nut, rice bran and cotton seed oils which were extracted from the seeds with hexane using Soxhlet apparatus.

Assay of enzymic activity. The proteolytic activity in the culture filtrate obtained at 72 hrs incubation was assayed by the method of Oshima [8]. Culture filtrates of higher protease activity were suitably diluted with distilled water prior to estimation. One unit of proteolytic activity was defined as when 2.5 ml of the culture filtrate completely hydrolyses 5 ml of 0.5% casein solution in one hr. at 40°.

Effect of non-nitrogenous carbon sources. The effects of changing carbon source on enzyme production is shown in Table 1. Among the various monosaccharides listed, only fructose gave slightly better enzyme formation (13.4 units/ml) than original medium (10.5 units/ml). Su and Liu [1] also reported that fructose resulted in better production of protease by *Aspergillus wentii*. Ustyuzhanina *et al.* [9] have observed that among the different carbon sources listed for the growth of *A. oryzae* for the production of protease, fructose promoted better protease synthesis. Of the disaccharides evaluated, lactose and maltose were found to be slightly better carbon sources than glucose for the protease formation and gave the enzyme yield of 11.6 and 12.6 units/ml respectively. With a strain of *Bacillus subtilis*, Emtseva [2] obtained better yield of alkaline protease using maltose as the carbon source in the medium. Out of the polysaccharides tested, starches were found to be better carbon sources for the production of the alkaline protease. Maize starch gave better enzyme yield (14.5 units/ml) than rice starch (12.6 units/ml) as well as potato starch (11.2 units/ml). Glycerol has been found to be the best carbon source for the growth of *Colletolrichum capaici* [3] but in our study with *A. oryzae* glycerol did not result in good growth of mold and there was a low yield of enzyme. Ethyl alcohol was poor carbon source for the enzyme formation in the present study. Methyl alcohol was inhibitory to mold growth as well as to the enzyme formation. Sodium acetate and lactic acid were found to be poor producer of the protease. Suzuki and Nakao [4] used paraffin as non-sugar carbon source for the production of mold alkaline protease and obtained reasonably good yield of the enzyme. In our study with *A. oryzae*, we found paraffin poor carbon source for the enzyme production. Both cane and beet molasses were found to be the best producers of the protease among the non-nitrogenous carbon source evaluated. Cane molasses gave slightly better yield of enzyme than the beet molasses, (16.4 and 15.2 units/ml respectively).

Effect of nitrogenous carbon sources. The effect of various industrial by-products such as soybean flour, rice bran, wheat bran mustard seed cake was examined. The results of these experiments are given in Table 1. Among nitrogenous carbon sources tried, wheat flour, ground nut flour, wheat bran and soybean flour gave almost similar yield of the enzyme. The yield of the enzyme ranged from 11.8 to 14.0 units/ml. Soybean gave slightly higher yield of the protease which corresponds to the results reported by the previous workers using a strain of *Bacillus licheniformis* [5].

Su and Liu [1] obtained a good yield of enzyme, when they included rice bran in the medium by *A. wentii*.In the present study with *A. oryzae*, rice bran was also found to be good producer of the protease and gave 13.6 units/ml. Among

TABLE 1. EFFECT OF CARBON SOURCES ON THE PRODUCTION OF PROTEASE.

Carbon source	Protease units/ml*
Non-nitrogenous	
Arabinose	4.8
Fructose	13.4
Galactose	9.0
Mannose	7.5
Sorbose	2.4
Xylose	4.8
Sorbitol	8.4
Mannitol	6.0
Lactose	11.6
Sucrose	8.4
Maltose	12.6
Dextrin	3.0
Inulin	7.5
Starch (Maize)	14.5
Starch (Rice)	12.6
Starch (Potato)	11.2
Ethyl alcohol	3.6
Glycerol	2.4
Methyl alcohol	–
Paraffin liquid	2.4
Lactic acid	6.0
Molasses (Cane)	16.4
Molasses (Beet)	15.2
Nitrogenous	
Ground nut flour	12.2
Wheat bran	13.2
Soybean flour	14.0
Maize flour	12.0
Wheat flour	11.8
Rice bran	13.6
Mustard seed cake	11.5
Control	10.5

* Each result is the average of three observation.

nitrogenous carbon sources the lowest yield of protease was obtained with mustard seed cake (11.5 units/ml). The better yield of protease obtained with these industrial by-products as compared with the majority of non-nitrogenous carbon sources may be attributed to the presence of trace metals, vitamins, amino acids and proteinaceous matters which are usually present in these by-products [10-12].

From the above discussion (Table 1) it is clear that 3% molasses (among the non-nitrogenous carbon sources) and 3% soybean flour (among the nitrogenous carbon sources) were the best producers of the alkaline protease. In Table 2 the effect of different concentration of both cane molasses and soybean flour on the formation of alkaline protease by *A. oryzae* has been shown. All of the concentrations of cane molasses tried, 3% was found to be the optimal for enzyme synthesis. In case of soybean flour, the enzyme production went on increasing, with the rise in concentration till it was maximum (14.8 units/ml) when the concentration was 4%. On further increase in the amount of soybean flour the enzyme synthesis decreased.

Effect of vegetable oils. Previous workers [13] had observed that the addition of vegetable oils to the fermentation medium increased the yield of proteases by *A. oryzae*. In our study, various vegetable oils such as ground nut, olive, castor, cotton seed, coconut, linseed, soybean, sunflower, corn and rice bran were added to the fermentation medium to find their stimulatory effect on the production of protease. The original medium [6] giving 10.5 units/ml of the enzyme was kept the same as used for the study of carbon sources. Oils were added to the original medium at a concentration of 0.5 ml/ 25 ml. The results are given in Table 3. Most of the oils tried, showed stimulatory effect on the yield of the enzyme. Among these, ground nut oil produced highest stimulatory effect (15.6 units/ml) followed by corn oil (14.2 units/ml) and rice bran oil (13.4 units/ml). Olive, cotton seed and linseed oils showed similar stimulatory effect and gave the enzyme yield of 12.3, 12.6 and 12.5 units/ml respectively. Soybean and sunflower oils showed very little stimulatory effect and produced the enzyme 11.0 and 11.5 units/ml respectively. Coconut oil and castor oil did not show any stimulatory effect. The low yield of enzyme production in the case of coconut and castor oil may be attributed to the low contents of unsaturated fatty acids in them [13].

Recently Leuchtenberger *et al.* [14] have studied the effect of various vegetable and animal oils on the growth and biosynthesis of protease by Thermoactinomyces and observed that these oils stimulated the protease production. However, the extent of stimulation by each oil was different. Similar results have been obtained by us in our experiments with *A. oryzae*.

Effect of salts on the production of protease. Salts of different metals were added to the original medium [6] to study their effect on the production of alkaline protease. The salts were tried in different concentration of 5, 10, 25, 35, 50 and 100 mg/100 ml of the medium. The enzyme produced was estimated after 72 hrs incubation. The quantity and quality of various salts effected differently on the enzyme production (Table 4).

Among the various chlorides tested, sodium chloride and potassium chloride in concentration of 25 mg of the salts were found to be better promotors of the enzyme synthesis, (17.4 and 14.2 units/ml respectively). On the other hand, calcium chloride and barium chloride gave maximum enzyme formation 14.4 and 11.5 units/ml respectively at 35 mg and 10 mg of the salts respectively. The results obtained by us with sodium chloride are in agreement with Narzymaski and Boguslaw [15] who observed that the addition of sodium chloride to the

TABLE 2. EFFECT OF DIFFERENT CONCENTRATIONS OF MOLASSES AND SOYBEAN FLOUR ON THE PRODUCTION OF PROTEASE.

Carbon source	Amount added gm/100 ml	Protease units/ml.*
	1	10.6
	2	11.5
Molasses	3	16.4
	4	15.6
	5	14.2
	1	8.5
Soybean flour	2	13.6
	3	14.0
	4	15.2
	5	13.4
Control	–	10.5

* Each result is the average of three observations.

TABLE 3. EFFECT OF OILS ON THE PRODUCTION OF PROTEASE.

Oil	Protease Units/ml.*
Ground nut	15.6
Corn	14.2
Rice bran	13.4
Olive	12.3
Cotton Seed	12.6
Linseed	12.5
Soybean	11.5
Coconut	11.0
Castor	10.8
Control	10.5
Sunflower	11.5

*Each result is the average of three observations.

TABLE 4. EFFECT OF SALTS ON THE PRODUCTION OF PROTEASE.PROTEASE (UNITS/ml) *

Salt	Amount of salts added per 100 ml					
	5mg	10mg	25mg	35mg	50mg	100mg
NaCl	8.8	13.2	17.4	14.6	12.8	11.6
KCl	9.6	12.4	14.2	12.8	11.4	11.0
$CaCl_2$	7.5	10.7	13.4	14.4	12.8	12.0
$BaCl_2$	8.4	11.5	10.8	10.5	10.2	9.8
KBr	8.8	11.5	9.8	8.6	8.2	8.0
K I	7.6	9.4	9.0	8.7	8.2	7.4
$NaCO_3$	8.5	10.4	9.8	9.3	9.0	8.6
K_2CO_3	7.9	10.2	9.5	9.1	8.6	8.2
$BaCO_3$	6. 0	9.8	9.0	9.0	8.8	8.0
Control	10.5 units/ml.					

* Each result is the average of three observation.

medium stimulated the production of the protease by *A. oryzae* and *A. niger*, 10 mg concentration of potassium bromide showed only slight increase in the yield of the enzyme. Potassium iodide was found to give low yield of the enzyme at all concentrations. The addition of carbonates of sodium, potassium and barium in concentration of 10 mg, has no material effect on the synthesis of the enzyme. However, in higher concentration these salts decreased the enzyme formation.

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