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SHAKE FLASK STUDIES ON THE PRODUCTION OF RAW STARCH HYDROLYZING AMYLOLYTIC ENZYMES BY *ASPERGILLUS NIGER* PCSIR-10

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Raw starch hydrolyzing amylolytic activity, produced by *Aspergillus niger* PCSIR-10, in the shake flask was investigated. The activity in the basal medium was 0.32 IU/ml and it increased to 6.0 IU/ml when the medium was supplemented with either corn-steep liquor (CSL), or corn starch or metal ions such as calcium and magnesium. The optimum pH, temperature and fermentation period were 3.5, 30° and 48 hrs, respectively.

Key words: Raw starch, Gelatinised, Saccharifying.

Introduction

The conventional method of conversion of starch into maltose and glucose is an energy intensive process. In view of the present energy crisis, alternate processes for the hydrolysis of raw starch into fermentable sugars at ambient temperature are being explored. Amylolytic enzymes are being extensively used for this purpose [1-8].

In the previous paper [1] techniques for the isolation and identification of *Aspergillus* sp., capable of hydrolyzing raw starch were reported. Different conditions for optimum production of enzyme by solid substrate fermentation were also discussed. This paper deals with the studies on the optimisation of culture conditions for the production of amylolytic enzymes by submerged fermentation.

Materials and Methods

Materials. Starch from corn, wheat, rice, potato, sweet potato and sago of commercial grade, were purchased from the local market. Soluble starch from E. Merck Co., Germany, peptone, tryptone and yeast-extract from Difco Laboratories, England, corn-steep liquor (CSL) from Glaxo Laboratories, Ferozepur Road, Lahore and milk casien was purchased from M/s BDH.

Micro-organism. The micro-organism used in this study was a strain of *Aspergillus niger* PCSIR-10, isolated from the local habitat. The stock cultures were maintained on potato-dextrose agar medium. The cultures were incubated at 30° for 5-7 days for maximum sporulation and then slants were kept in the refrigerator.

Fermentation technique. The basal medium having the following composition (gm/l) was used for enzyme production: soluble starch 10, peptone 10 and yeast extract 2. Raw starch whenever used was always sterillised separately in a hot air dry oven at 140° for a period of 2 hrs and then aseptically mixed with the pre-sterilised peptone yeast extract medium.

Optimum culture medium consisted of (g/l) corn starch 15, corn steep liqour 35, yeast extract 2.0, NaCl (3.0), KH_2PO_4 (1.0), $CaCl_2.2H_2O$ (0.3) and $MgSO_4.7H_2O$ (0.4), at pH, 3.5. 50ml of this culture medium was taken in a 250ml conical flask and inoculated with a loopful of organism from a slant of *A. niger* PCSIR-10. Cultures were then incubated at 30±2° for a period of 48 hrs on a rotary shaker kept at 300rpm. After the completion of incubation period, the broth was filtered through an ordinary filter paper. The filtrate thus obtained was centrifuged at 3000xg and then analyzed for various enzymic activities.

Analytical methods. Raw starch digesting activity gelatinized starch saccharifying activity and alpha-amylase activity was analyzed according to earlier methods [2].

Raw starch digesting activity. It was measured according to the methods described in our previous publication [1].

Gelatinized starch saccharifying activity. This was assayed using a substrate solution which consisted of 1.0ml of 2.0% soluble starch solution and 1.0ml of 0.1M acetate buffer (pH 3.5). The reaction was stopped by heating the flask in a boiling water bath for 5 mins. The amount of reducing sugars released was determined by Somogy-Nelson Method [13] with glucose as standard.

Enzyme unit. One unit of enzyme activity was defined as the amount of enzyme which can bring about one micromole of glucosidic bond cleavage per minute under the respective reaction conditions.

Alpha amylase activity. This was assayed by the following method. 1.0ml of enzyme solution was added to 1.0ml of 1.0% soluble starch solution in o.1M acetate buffer (pH 6.6) and the mixture was incubated at 35° for a period of 10 mints. Then 1.0ml of the mixture was taken out and added to 10ml of o.1N HCl. From this mixture 0.5ml was withdrawn and added to 10ml of a mixture which originally consisted of 0.005% iodine and 0.05% potassium iodide. The photometrical absorbance was noted at 660nm.

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Enzyme unit. One unit of alpha amylase activity was defined as the amount of enzyme which will produce a 10% fall in the intensity of blue colour of amylose-iodine complex under the conditions mentioned above.

Results and Discussion

Effect of various carbohydrate sources on enzyme production. The effect of addition of various carbon sources on the production of enzyme is depicted in Table 1. Raw starches were found to be more effective as compared to the gelatinized starches. The starches of corn, wheat and sweet potato proved to be the most effective inducers of raw starch digesting activity i.e. 0.98, 0.56 and 0.48 IU/ml were produced respectively. Other carbohydrates such as rice, potato and sago were less effective i.e. 0.35, 0.32 and 0.22IU/ml were produced respectively. The data on gelatinized starch saccharifying glucoamylase activity is in general confirmity with the raw starch. Greater activity was, however, observed in case of corn, wheat and sweet-potato starch. These activities, in general, were 5-6 times higher than the raw starch digesting activity. Similar results were obtained in case of gelatinized starch.

Alpha amylase activity and final pH of the medium were also studied. The maximum production of Alpha-amylase activity (2.42IU/ml) was recorded in case of wheat starch. Final pH of the medium, in most of the cases, either remained neutral or was slightly acidic.

The effect of various concentration of carbohydrate sources, i.e. wheat, corn and sweet potato, selected on the basis of better enzyme production, were examined (Fig. 1). The maximum amount of enzyme activity (0.98IU/ml) was produced in case of corn starch at a concentration of 1.5%.

The decreased enzymic activity observed in case of sago and potato starch may be attributed to the presence of a hard coat around the starch granules, thus making the granule digestion difficult [12].

Effect of various nitrogen sources on the enzyme production. The effect of addition of various organic, inorganic and complex nitrogen sources to the basal medium was investigated (Table 2). The nitrogen sources were added at a concentration of 1.0gm nitrogen/l of the culture medium. Sodium nitrate, polypeptone and corn steep liquor produced better results i.e. 1.13, 1.88 and 2.00IU/ml of raw starch digesting activity were produced respectively. The data on gelatinized starch saccharifying activity was in general confirmity with that of raw starch. These activities, were however, 5-6 times higher than the raw starch.

The three nitrogen sources, which produced better results, were then compared at various concentrations to select the most effective nitrogen source. It is evident from Fig. 2, that corn steep liquor, (CSL) at a concentration of 3.5% produced 2.40IU/ml of enzyme activity and thus proved to be the most effective source of nitrogen. Similarly, the maximum amount of Alpha-amylase activity (3.32 IU/ml) was recorded in case of CSL, while the final pH of the medium, in most of the cases, either remained neutral or slightly acidic (Table 2).

TABLE 1. EFFECTS OF VARIOUS CARBOHYDRATES ON ENZYME PRODUCTION.

Carbohydrate (1.0%)	Final pH	Glucoamylase activity (IU/ml)		Alpha-amylase activity (U/ml)
		Raw*	Gelatinized**	
Raw starch				
Corn	6.8	0.98*	4.90	2.31
Wheat	7.0	0.56	2.82	2.42
Rice	6.5	0.35	1.75	1.00
Potato	6.3	0.30	1.60	1.70
Sweet Potato	7.2	0.48	2.41	2.35
Sago	7.1	0.22	1.12	0.74
Gelatinized starch				
Corn	6.4	0.82	4.10	1.16
Wheat	6.7	0.52	2.53	1.35
Rice	5.5	0.36	1.82	0.84
Potato	7.2	0.31	1.50	1.13
Sweet Potato	6.9	0.48	2.44	1.42
Sago	7.0	0.27	1.36	1.92
Soluble starch	7.1	0.32	1.51	1.37

Cultivation carried out at 30±2° for 48 hrs on a rotary shaker at 300 rpm. Medium = 1.0% polypeptone + 0.2% yeast extract + carbohydrate 1.0%.
* Raw starch digesting activity. ** Gelatinized starch saccharifying activity.

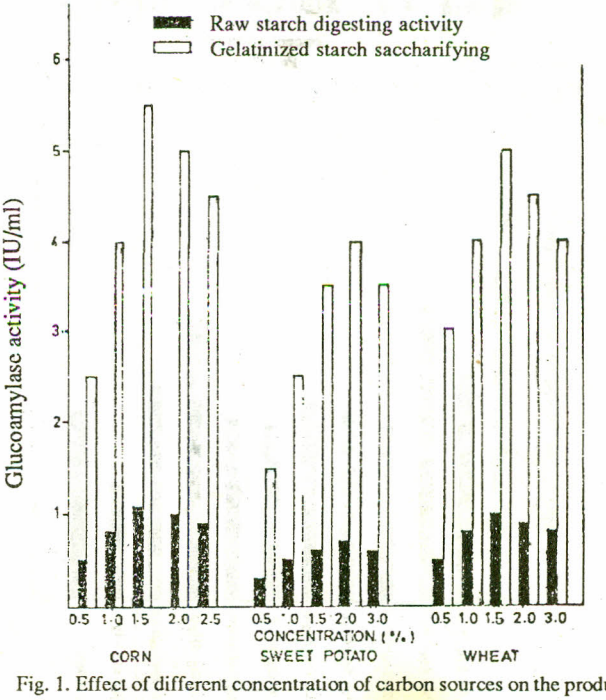

Fig. 1. Effect of different concentration of carbon sources on the production of enzyme.

Stimulative effect of CSL on enzyme production, during submerged fermentation, has also been reported by Yamada and Tamoda [9]. Chemical analysis of CSL shows that it is a rich source of minerals and vitamins, in addition to ready hydrolysable proteins [10].

Effect of various metal ions on enzyme production. The effect of addition of various metal ions to the basal medium was examined (Fig. 3). The metal ions were added at a concentration 1.0 mM. These included Fe^{+3}, Mg^{+2}, Cu^{+3}, Zn^{+2}, Mn^{+2}, Al^{+3}, Co^{+2}, Ni^{+3}, $Ba^{+2}$and Ca^{+2}, in the form of their respective salts. Figure. 3 shows that most of the metal ions,

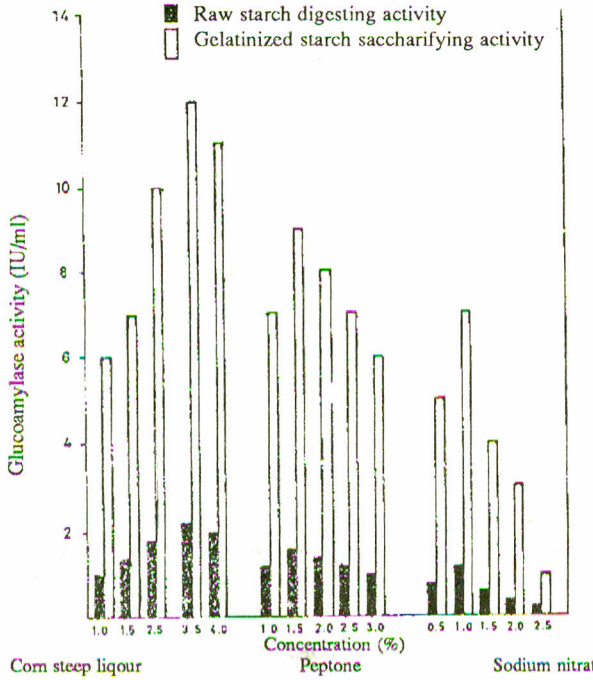

Fig. 2. Effect of different concentration of nitrogen sources on the production of enzyme.

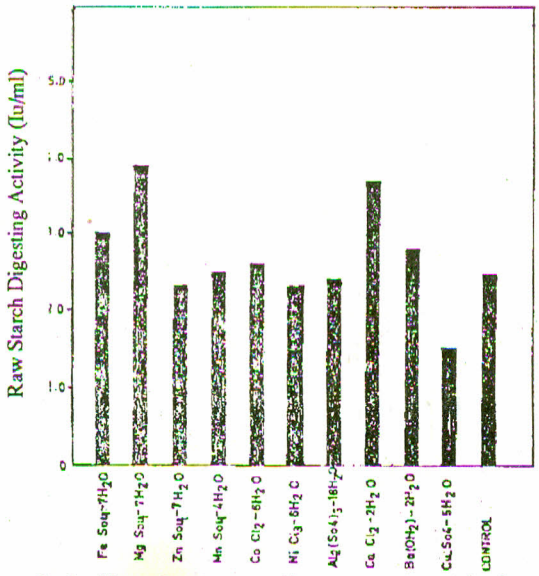

Fig. 3. Effect of various metal ions on enzyme production.

enhanced the enzyme activity except for $CuSO_4.5H_2O$. Toxic effects of copper on fungal metabolism have also been reported by Ishigami *et al.* [2]. It was also notice that Fe^{+3}, Mg^{+2} and Ca^{+2} were comparatively more effective in increasing the enzyme activity i.e. 3.0, 3.9 and 3.7 IU/ml were produced, respectively.

In another experiment the effect of separate addition of Ca^{+2} and Mg^{+2} to the culture medium, at various concentrations was also studied (Table 3). The best results (4.38 IU/ml) were obtained when Ca^{+2} and Mg^{+2} were added simultaneously to the culture medium at a concentration of 2.0 and 1.5 mm, respectively (this corresponds to 0.029 and 0.037%). However for large scale used the amount was modified to 0.03% and 0.04% respectively. The stabilizing effect of calcium ions on amylases has been reported by Hsiu *et al.* [11]. They reported that calcium does not participate directly in the formation of enzyme-substrate-complex but rather holds the enzyme mole-

TABLE 2. EFFECT OF VARIOUS NITROGEN SOURCES ON ENZYME PRODUCTION.

Carbohydrate (1.0%)	Final pH	Glucoamylase activity (IU/ml)		Alpha-amylase activity (U/ml)
		Raw	Gelatinized	
In-organic				
$NaNO_3$	7.0	1.13	5.65	2.41
NH_4NO_3	6.3	0.86	4.83	2.31
$(NH_4)_2SO_4$	6.5	0.84	4.22	1.19
$(NH_4)_2HPO_4$	7.1	0.67	3.16	1.20
Organic				
Peptone	6.8	1.88	9.40	1.45
Tryptone	7.2	1.62	8.11	3.05
Milk casien	7.0	1.34	6.72	2.73
Yeast extract	7.1	1.46	5.45	2.15
Complex				
CSL	7.2	2.00*	10.0	3.32
Wheat bran	6.6	1.07	5.35	2.10
*Soyabean meal	6.2	1.78	8.90	3.13
*Cotton seed meal	6.3	1.26	6.32	2.42

*Defatted soyabean meal and cotton seed meal.
Cultivation carried out at 30±2° for 48 hrs on a rotary shaker at 300 rpm.
Medium = 1.5%corn starch + 0.2% yeast extract + nitrogen 0.1%.

TABLE 3. COMBINED EFFECT OF CALCIUM AND MAGNESIUM ON THE ENZYME PRODUCTION.

Mg^{+2} Concentration (mM)	Raw starch digestion activity IU/ml Ca^{+2} concentration (mM)				
	0.5	1.0	1.5	2.0	3.0
0.5	1.10	1.82	2.09	2.20	2.11
1.0	2.63	2.87	2.92	3.00	3.17
1.5	3.21	3.86	3.95	4.38*	3.60
2.0	2.31	2.57	2.73	2.86	3.00
2.5	3.02	3.25	3.50	3.67	3.22

Culture medium: 1.5% corn starch + 3.5% CSL + 0.2% yeast-extract + Calcium + Magnesium.

cule in the correct conformation for activity and maximum stability.

Effect of initial pH of the medium on the production of enzyme. Effect of initial pH of the medium on enzyme production was investigated. Different pH values ranging from 2.5 to 4.5 were tried out. Maximum amount of enzyme activity (5.50 IU/ml) was produced at 3.5 pH. A rapid decrease in the growth rate and rate of enzyme synthesis was observed at a pH below 2.5 (Fig. 4).

Effect of cultivation temperature on the enzyme production. Effect of temperature on enzyme production was studied in 250ml flasks containing 50ml of the culture medium. The flasks were incubated on a temperature conctrolled rotary

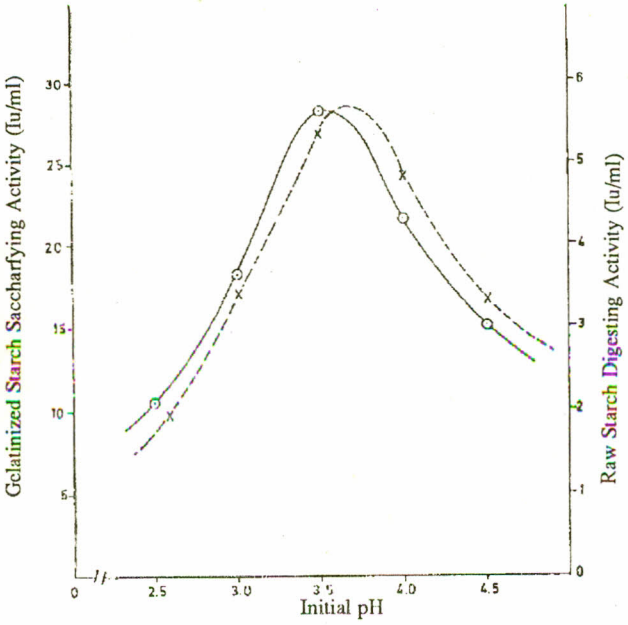

Fig. 4. Effect of initial pH of the medium on enzyme production.

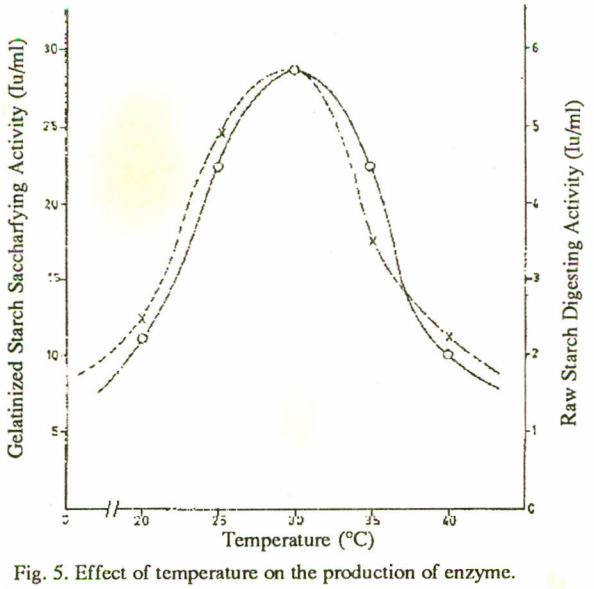

Fig. 5. Effect of temperature on the production of enzyme.

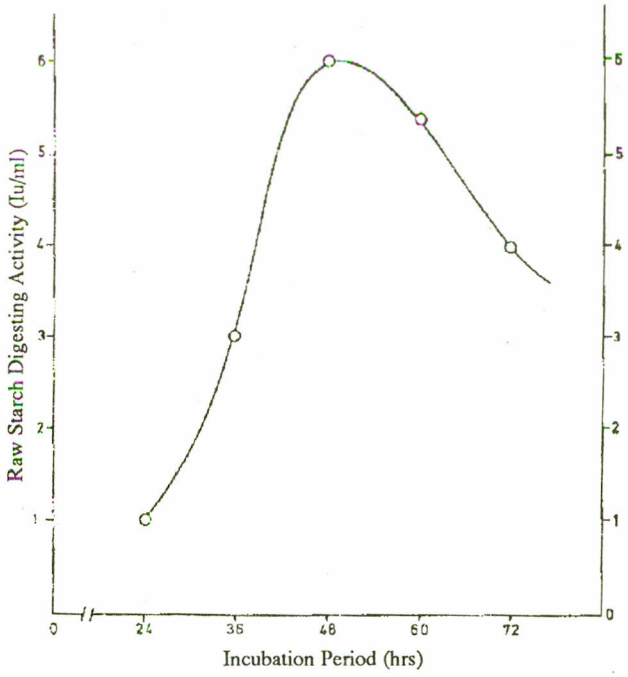

Fig. 6. Time course of enzyme synthesis by *Aspergillus niger* PCSIR-10.

shaker at different temperatures ranging from 20 to 40°, for a period of 48 hrs. Figure 5 shows that maximum amount of enzyme activity (5.75 IU/ml) was produced at 30°. Optimum enzyme activity was observed between 25 and 35°, while at a temperature below 20° there was a rapid decrease in the rate of enzyme synthesis.

The study of fermentation period. The flasks containing the culture medium were incubated at 30±2° on a rotary shaker kept at 300 rpm for a period of three days. An aliquot of the cultural broth was withdrawn after every 12 hrs and was analyzed for enzyme activity. Maximum amount of enzyme activity (6.0 IU/ml) was produced after an incubation period of 48 hrs. Further increase in the fermentation period did not improved the rate of enzyme synthesis (Fig. 6).

Enzyme production under optimum culture conditions. The composition of optimum culture medium for enzyme production is given in materials and methods. Slight modifications such as addition of NaCl and KH_2PO_4 were also made. The amount of enzyme produced under optimum culture conditions was 6.0 IU/ml.

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