EFFECT OF NITROGEN SOURCES ON THE PRODUCTION OF α-AMYLASE

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Aspergillus oryzae IMI 17299 was used to find out the effect of nitrogen sources on the production of α-amylase. The organism was grown in basal starch-ammonium sulphate-salts medium, in 300-ml flasks on a rotary shaker at 30°C. α-Amylase activity in the culture filtrate was determined spectrophotometrically. Ammonium sulphate in the medium was replaced by various inorganic, organic nitrogenous compounds and industrial by-products. Of the various inorganic nitrogenous compounds used, ammonium sulphate gave maximum amylase production 9.36 units/ml, NH_4NO_3, $NaNO_3$, K NO_3, gave 8.87, 6.05, 7.09 units/ml culture filtrate, respectively. In the case of organic compounds and by-products, maximum amylase production was achieved at 96 hr. Peptone gave maximum amylase production 15.80 units/ml; caseitone and yeast extract gave 14.44 and 14.80 units/ml culture filtrate, respectively. Urea behaved like inorganic compounds and showed low amylase production 7.70 units/ml. Cornsteep liquor, an industrial by-product, gave the highest amylase production (22.26 units/ml) among all nitrogenons compounds tried in these experiments. Distiller soluble showed poor amylase production.

Introduction

Due to great demand of α-amylase enzyme in industries like pharmaceutics, bakery, food, fruit juices, starch and syrups,[1] the production of α-amylase by *Aspergillus oryzae* was undertaken with a view to exploiting the possibilities of production on large scale. Several workers[2–10] have studied the effect of different sources of nitrogen on production of α-amylase by microorganisms. In the present studies we have selected submerged culture method as it has been reported useful by various workers.[1,3,6,10–19]

Although the microorganisms *Aspergillus oryzae* and *Aspergillus niger* have been found to produce α-amylase,[1,3–6,8] the one *Aspergillus oryzae* already employed for industrial purposes has been selected in the present investigation.

Materials and Methods

Organism.—The microorganism used in this study was a strain of *Aspergillus oryzae* (IMI–17299 Diastase) and the stock cultures were maintained on Czapek Dox agar [20] media in 150×25 mm test tubes.

Media.—Czapek Dox agar media with the following components (per litre of solution) was used: sucrose, 30 g; $NaNO_3$, 2.0 g; KH_2PO_4, 1.0 g; $MgSO_4$, 0.50 g; KCl 0.50 g; $FeSO_4$, 0.01 g; agar agar, 25.0 g.

The organism was grown on Meyrath[21] medium with the following composition (per litre solution): starch, 40 g; $(NH_4)_2SO_4$, 8.0 g; Na_2HPO_4, 35.5 g; KH_2PO_4, 34.0 g; $MgSO_4$, 0.30 g., $FeCl_3$, 0.003 g. The media was prepared by dissolving minerals in 650 ml deionized water; starch was suspended in about 100 ml cold water and was poured into boiling mineral solution. Boiling was continued for 2 min; it was then cooled, filtered and the volume made up to 1 l. The pH of the media was self adjusted to 6.8. The filtration of the medium was found necessary as it removes compounds which are precipitated during the media preparation. It has been reported that inhibiting substances lower the amylase production and produce greater mycelium dry weight. 24.0 ml of the media was dispensed into 300 ml flasks, plugged with cotton wool and autoclaved at 15 lb/in^2, cooled at room temperature and then inoculated with 1.0 ml portion of inoculum having equal number of spores each time. These flasks were incubated at 30°C on rotary shaker (120–125 rpm), and removed at different intervals for the assay of amylase activity.

Cultures of *Aspergillus oryzae* obtained by growing the organism in basal medium or in which certain other compounds were included as a sole source of nitrogen in place of ammonium sulphate, were harvested at different intervals pressed with rubber bung to get maximum exudate and the amylase activity was then examined in the filtrates. Mycelium was washed with deionized water and dried to a constant weight at 100–105°C.

Assay of Enzymic Activity.—After having recovered the mycelium the filtrates were examined for α-amylase activity. The enzymic assay was carried out according to the method of Fernandez and Sobel.[22] 0.2 ml culture filtrate was added to 3.5 ml substrate in duplicate tubes and incubated at 37°C for 30 min. The tubes were removed immediately and 1.0 ml arsenious acid–potassium iodide solution and then 0.5 ml ethylamine hydrochloride solution was added. Tubes were then placed immediately in a boiling water bath for 15 min. A standard and blank was also kept simultaneously.

After 15 min, the tubes were removed from the water bath one at a time and immediately 0.5 ml 20% NaOH was added to each tube while still hot. The tubes were cooled at room temperature and absorbancy was read on SP-600 spectrophotometer at wavelength 535 mμ.

Results and Discussion

Various inorganic compounds, $(NH_4)_2SO_4$, NH_4NO_3, KNO_3 and $NaNO_3$, and organic compounds (caseitone, peptone, yeast extract and urea) and industrial by-products (cornsteep liquor and distiller soluble) were tried as sole source of nitrogen in the basal medium. The amylase production in early stages of growth was negligible, up to 48-hr incubation. Thereafter, amylase activity in the culture medium was determined every 24 hr. In the case of inorganic compounds (Table 1) there was a little growth and amylase production up to 72 hr showed a rapid rise and reached maximum at 120 hr and then a sharp decrease was observed at 144 hr. Sodium nitrate, however, showed maximum amylase production at 96 hr and decreased afterwards. In the case of organic compounds (Table 2) growth and amylase production was appreciable at 48 hr and showed rapid rise which reached maximum at 96-hr incubation and decreased thereafter. The behaviour of urea was similar to that of inorganic nitrogen compounds. There was a little difference noted in amylase production at 72 hr and 96 hr. Therefore, with organic compounds, maximum amylase production can be had at 72 hr as compared to 120-hr incubation in the case of inorganic nitrogen compounds. Better results of amylase production were achieved in the case of organic nitrogen sources as compared to inorganic nitrogen sources.

Initially, detailed examination was made of the effect of different concentrations (0.050, 0.085, 0.170, 0.225, 0.340%N_2) of ammonium sulphate on the growth and production of α-amylase. When this compound was included in the basal medium as a sole source of nitrogen there was a gradual increase in the growth with the increasing amount of N_2 used. The amylase production reached its maximum at 120 hr (Table 1). These results are in agreement with the studies made by Tanabe *et al.*[2] and Murota *et al.*[8]

Amylase activity of the culture medium increased as the concentration of N_2 was raised from 0.050 to 0.170% and then there was a gradual fall in amylase activity, when nitrogen concentration was further raised to 0.225%N_2. A maximum amylase activity (9.36 units/ml) was obtained with 0.170%N_2 (Table 1).

Since the active growth and production took place between the concentration range 0.085 to 0.225%N_2, in other experiments in which other nitrogenous compounds were used in the basal medium the concentrations selected were 0.140, 0.170 and 0.200%N_2.

Of the various inorganic sources used in the basal medium $(NH_4)SO_4$ was the one which gave maximum amylase production (9.36 units/ml) and ammonium nitrate was the next having amylase production of 8.87 units/ml. Results obtained with ammonium sulphate are similar to those observed by Tanabe *et al.*[2] and Murota *et al.*[8] There was very little difference 0.490 units/ml between the amylase units produced with some 0.170%N_2 concentration of $(NH_4)_2SO_4$ and NH_4NO_3 at 120 hr. This similarity in amylase production can be explained as suggested by Cockrane.[23] Ammonium nitrate contains both reduced and oxidised forms of nitrogen (NH_4) (NO_3). When NH_4 group is completely assimilated, the NO_3 group gets reduced and its utilisation starts. This mechanism provides regular supply of nitrogen in the medium for the synthesis of enzyme. Tanabe *et al*,[2] Asai *et al*,[5] Murota *et al.*[8] using strain of *Aspergillus oryzae* have also found NH_4NO_3 useful nitrogen source for the production of α–amylase. Shu and Blackwood[2] and Dunn *et al.*[6] regarded NH_4NO_3 a poor source of amylase production using *Aspergillus niger*. The amylase activity was low with $NaNO_3$ and KNO_3. The maximum amylase units obtained with the former was 6.05 units/ml while the latter gave 7.30 units/ml.

Several workers have reported that various organic nitrogenous compounds such as caseitone, yeast extract and peptone stimulate amylase production in microorganisms when included in the medium as source of nitrogen.[2,3,5,6,8,16] The stimulatory effect of these compounds was tried with comparatively lower concentration and encouraging results have been obtained. Amylase production 14.44 units/ml was achieved with caseitone at concentration of 0.170%N_2. Further increase in the %N_2, however, did not improve the amylase production. With yeast extract, maximum amylase production (14.50–14.80 units/ml) was obtained with 0.240%N_2. There was a little difference in amylase units (0.60/ml) when 0.32%N_2 was used. Therefore, 0.24%N_2 concentration can be regarded as suitable for amylase production. In the case of peptone, maximum amylase production (15.24 units/ml) was obtained with 0.085%N_2. Almost similar results were obtained with 0.170%N_2. Amongst these three compounds used, peptone has been found to have most stimulating effect on amylase

TABLE 1.—EFFECT OF INORGANIC NITROGEN SOURCES ON THE PRODUCTION OF α-AMYLASE.

Source of nitrogen	Nitrogen %	α-amylase units per ml					Mycelium dry weight (g/l)				
		48 hr	72 hr	96 hr	120 hr	144 hr	48 hr	72 hr	96 hr	120 hr	144 hr
Ammonium sulphate	0.050	0.67	1.70	2.50	3.41	2.80	0.68	0.96	1.20	1.80	1.85
	0.085	0.97	2.56	4.62	5.70	5.10	0.92	1.36	1.88	2.24	2.34
	0.170	2.11	4.08	7.14	9.36	8.50	1.40	2.60	3.10	4.30	4.40
	0.225	3.23	4.67	5.34	8.20	7.60	1.70	3.00	3.80	5.40	5.50
	0.340	4.44	4.94	5.48	5.63	5.20	1.90	3.30	3.90	5.80	6.10
Ammonium nitrate	0.140	2.83	3.54	6.30	6.72	6.15	1.50	1.90	2.70	3.10	3.30
	0.170	3.48	6.08	8.00	8.87	8.25	1.80	2.60	3.40	4.30	4.50
	0.200	4.14	6.47	7.35	8.19	7.80	2.20	3.00	3.80	4.50	4.90
Sodium nitrate	0.140	2.18	3.83	4.83	5.25	5.00	0.88	1.24	1.80	1.88	1.62
	0.170	2.08	3.48	5.91	6.05	5.60	0.96	1.30	2.00	2.08	1.62
	0.200	3.14	4.22	5.88	6.30	5.50	1.20	1.60	2.20	2.38	2.12
Potassium nitrate	0.140	3.96	5.88	6.30	6.60	6.10	1.10	2.20	2.70	3.40	3.10
	0.170	4.17	4.34	5.40	7.09	6.70	1.30	2.48	3.15	4.25	3.80
	0.200	4.20	5.88	7.14	7.30	7.00	1.50	2.80	3.60	4.70	4.25

TABLE 2.—EFFECT OF ORGANIC NITROGEN SOURCES ON THE PRODUCTION OF α-AMYLASE.

Source of nitrogen	Nitrogen %	α-amylase units per ml					Mycelium dry weight (g/l)				
		48 hr	72 hr	96 hr	120 hr	144 hr	48 hr	72 hr	96 hr	120 hr	144 hr
Caseitone	0.050	3.13	4.59	4.72	4.30	3.75	1.44	2.24	2.60	2.76	2.60
	0.100	7.93	10.23	10.60	9.70	9.18	2.24	3.28	3.72	3.92	3.64
	0.140	9.10	12.25	12.60	12.00	11.10	3.20	4.60	5.00	5.60	5.20
	0.170	9.10	14.44	14.25	13.40	12.80	3.40	5.40	6.40	6.60	6.00
	0.200	9.70	14.23	14.44	13.80	13.20	3.68	5.20	5.80	6.40	6.20
Yeast extract	0.080	7.60	10.00	10.44	9.60	8.60	2.80	3.20	4.20	4.40	4.00
	0.160	8.60	10.92	11.20	10.45	9.45	3.00	3.60	4.80	5.20	5.00
	0.240	10.44	14.50	14.80	14.20	13.10	3.60	4.80	5.60	6.20	5.80
	0.320	10.85	15.10	14.80	14.40	13.60	3.90	5.20	5.90	6.40	6.20
Peptone	0.017	4.34	5.42	5.84	5.50	5.20	2.56	3.60	3.92	4.40	4.60
	0.085	10.44	15.24	15.40	14.20	13.40	3.24	5.10	5.98	6.20	6.50
	0.170	11.20	15.28	15.80	14.60	13.25	3.82	5.80	7.88	8.20	8.60
Urea	0.140	2.26	2.31	4.51	6.70	4.70	1.30	2.00	3.40	3.80	4.40
	0.170	3.28	4.64	5.60	7.30	5.40	1.70	2.40	3.20	4.40	4.80
	0.200	3.35	4.83	6.56	7.70	5.20	1.80	2.60	3.80	4.20	4.80

TABLE 3.—EFFECT OF INDUSTRIAL WASTES AS NITROGEN SOURCES ON THE PRODUCTION OF α-AMYLASE.

Source of nitrogen	Nitrogen %	α-amylase units per ml					Mycelium dry weight (g/l)				
		48 hr	72 hr	96 hr	120 hr	144 hr	48 hr	72 hr	96 hr	120 hr	144 hr
Cornsteep liquor	0.14	10.44	15.70	16.12	14.13	13.20	2.80	3.60	3.90	4.10	3.70
	0.17	12.50	20.20	22.26	20.48	18.70	3.20	3.60	4.20	4.50	4.10
	0.20	11.50	18.80	20.20	19.40	18.50	3.52	3.80	4.40	4.60	4.30
Distiller's soluble	0.14	1.46	1.56	1.42	1.34	0.96	1.60	1.80	2.00	20.00	1.80
	0.17	1.98	2.10	1.92	1.66	1.04	2.00	2.20	2.32	2.26	2.00
	0.20	1.87	2.50	2.25	1.86	1.12	2.40	2.70	2.88	2.56	2.56

production. We have obtained better α-amylase production than that reported by previous workers.[3,6,16] Urea was an exception whose behaviour was almost similar to that of inorganic compounds.

This greater stimulation by caseitone, yeast extract, and peptone may be due to the presence of trace metals, vitamins, amino acids, and polypeptides, which might not be present in other compounds supplemented in the media.[2,3,6,9,16]

Probably these low molecular weight nitrogenous compounds contribute to the enhanced synthesis of enzyme, α-amylase. Comparatively a greater stimulation (22.26 units/ml) was obtained with cornsteep liquor with 0.170% N_2 concentration at 96-hr incubation (Table 3). Recently Yamada and Tamoda[24] have used cornsteep liquor as nitrogen source for the production of α-amylase. This great increase in amylase production might be due to 'phytic acid' present in it.[25] Prior to this, the stimulatory effect of phytic acid on amylolytic enzymes was shown by Dunn *et al.*[16] Other factors responsible for the increase in production of α-amylase are vitamis, trace elements and amino acids.[3,6,9,16] All these substances have been reported to be present in cornsteep liquor.[26,27]

A poor growth and amylase production with distiller soluble shows that probably it does not contain the above-mentioned nitrogenous compounds and some inhibitory substances could be present which cause low growth and amylase production.

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