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BIOSYNTHESIS OF ENZYMES BY SOLID SUBSTRATE FERMENTATION*

Part II. Production of Alpha-Amylase by *Bacillus subtilis*

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Production of alpha-amylase by the locally isolated cultures of *Bacillus subtilis* was studied using wheat bran as solid substrate in conical flasks. The selected culture was improved by UV irradiation. Partial replacement of wheat bran by other substrates such as rice husk, maize bran, peanut meal or penicillium-waste mycelium was also investigated for enzyme formation. Of all the substrates wheat bran was found to be an ideal for enzyme synthesis.

INTRODUCTION

In continuation of our studies regarding the use of wheat bran as solid substrate for the synthesis of cellulase [1] and amyloglucosidase [2] the present work describes the production of alpha-amylase by *Bacillus subtilis*. The wheat bran or brans of other cereals were also evaluated for enzyme formation by solid substrate fermentation. The wheat bran or brans of other cereals were also evaluated for enzyme formation by solid substrate fermentation. The surface culture or the Koji process, using trays commonly used in Japan, requires a large surface area but other factors such as rates of agitation and aeration, foaming, contamination or pH control are not serious problems in fermentation in deep tanks. Microbial amylases are extensively used for starch thinning in glucose and beer manufacture, improving dough for bread making, desizing of textile and in pharmaceutical preparations. These enzymes are imported into Pakistan involving substantial amount of foreign exchange and the largest consumer in the country is the textile industry. Alpha-amylase of bacterial origin is thermostable and thus can withstand high temperature during desizing operation in the textile industry. Much work has been done on the production of microbial amylase by surface culture process using wheat bran as a substrate [3–14]. Cultures of *Bacillus subtilis* were isolated locally from the soil and the selected strains was further improved by mutation. Enzyme formation was carried out in 1l conical flasks containing cereal brans.

MATERIALS AND METHODS

Organisms and Growth. Fifty strains of *Bacillus* species were isolated after giving heat shock to suspension of soil, wheat flour or maize products in water. The bacterial cultures were maintained on nutrient agar medium consisting of (g/l): peptone 6.0, casein (hydrolyzate) 4.0, yeast extract 3.0, glucose 2.0, beef extract 1.5 and agar 25.0. The cultures were incubated at 37° for 48 hr and then stored in a refrigerator at 5°. All isolates were gram + ve spore-forming rods which were identified as *Bacillus* species using the criteria of Bergey's Manual of Determinative Bacteriology. The culture producing maximum enzyme was further identified as *Bacillus subtilis*.

Inoculum Preparation. A vegetative inoculum was employed in the present work. 100 ml of nutrient broth, as described earlier in 1l conical flask was sterilized at 121° for 15 min. After cooling the medium, it was inoculated from the agar slant aseptically. It was allowed to grow at 30° for 24 hr on a shaker (designed and fabricated at these Laboratories, rotating at 125 rev/min).

Fermentation Procedure. A mash of 20–50 g wheat bran or the bran of other cereals was prepared by adding 2 parts of diluent phosphate buffer (1.5 g KH_2PO_4 and 3.5 g K_2HPO_4/l) or otherwise stated to one part by weight of wheat bran in 1l conical flask. The mash was sterilized at 121° for 15 min. The sterilized mash was inoculated with 2 ml of the inoculum prepared as above and incubated at 30° for 48 hr.

Culture Improvement. A 24-hr old bacterial cell suspension was centrifuged aseptically and washed with saline water twice and resuspended in 0.9% saline solution. The suspension of cells, placed in a petri dish, was placed under UV lamp. The samples were taken at different time intervals and cultivated on nutrient agar and incubated at 37°

Preparation of Enzyme Extract. The fermented bran

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cake was shaken for 1 hr at 30° on a rotary shaker after the addition of 200 ml phosphate buffer (pH 7.00) as described earlier. To this mixture was added 1 ml 20% $CaCl_2.2H_2O$ solution for each 40 ml of the buffer. The wheat bran suspension was filtered using ordinary filter paper. The clear filtrate was assayed for enzyme activity.

Analytical Methods. Amylase activity was determined by the method of Fisher and Stein [15]. Enzyme solution (1 ml culture filtrate) was added to 1 ml 1% starch solution and incubated at 25° for 3 min. The reaction was terminated by the addition of 2 ml DNS (3,5-dinitrosalicylic acid) reagent (DNS, 5%). Colour due to the reducing sugar liberated was developed by heating the reactants in a boiling water bath for 5 min then rapidly cooling them to room temperature. The extinction value was determined at 550 nm spectrophotometrically using a 'spectra' by EEL Halstead Essese England.

One amylase unit liberates reducing sugar equivalent to 1.0 mg maltose hydrate under the conditions of assay. Calibration curve was also prepared using maltose 0.5 – 2.0 mg/ml.

RESULTS

Screening of Organisms. The data in Table 1 indicate the biosynthesis of alpha-amylase by the locally isolated cultures *of Bacillus subtilis* in wheat bran by the solid substrate fermentation process. The assay of enzyme was carried out 48 hr after inoculation with 24-hr old seed culture. The amount of the enzyme synthesized by the cultures No. 1, 2, 26 and 42 was 360, 370, 230 and 238 units alpha-amylase/g wheat bran respectively. For further studies, therefore, culture No. 2 was selected for the production of alpha-amylase and it was identified as *Bacillus subtilis* (PCSIR - 2).

Culture Improvement by Mutation. Bacillus subtilis PCSIR - 2 was exposed to UV irradiation. The data in Table 2 show the synthesis of alpha-amylase by *Bacillus subtilis* after irradiation for different time periods. Duplicate cultures of each isolate were examined for enzyme formation.

Culture PCSIR-29, isolated after irradiation for 40 min, produced maximum amount of alpha-amylase at 4000 units/g of wheat bran. Further irradiation did not result in any improvement in enzyme synthesis. *Bacillus subtilis* PCSIR - 29 was used for further improvement by exposing bacterial cells to 0.1 – 1.0 mg/l of 6-mercaptopurine for different time periods (Table 3). Most of the isoated colonies did not show any improvement in enzyme formation. One colony isolated (PCSIR-20) after exposing bacterial cells to the chemical for 20 hr produced 5850 units of alpha-amylase/g wheat bran. The vegetative cells of the culture (PCSIR 20) was again exposed to chemical and UV radiation ultimately

Table 1. Production of alpha-amylase, 48 hr after inoculation, by locally isolated cultures of *Bacillas subtilis* in wheat bran by surface culture method.

Isolate No.	Wheat bran (units/g)	Isolate No.	Wheat bran (units/g)
1	360	26	230
2	370	27	27
3	60	28	11
4	30	29	12.5
5	115	30	44
6	70	31	34
7	60	32	25
8	65	33	24.5
9	115	34	3.5
10	90	35	14
11	50	36	5
12	95	37	4
13	85	38	50
14	73	39	56
15	64	40	47
16	52	41	46
17	65	42	238
18	35	43	42
19	128	44	33
20	127	45	44
21	38	46	187
22	31	47	137
23	33	48	93
24	40	49	85
25	50	50	105

(Table 4). Alpha-amylase synthesis was increased as compared with the parent culture. The culture No. 6 produced maximum amount of enzyme alpha-amylase 6300 units/g wheat bran. This culture was found to be unstable and after two or three generations the enzyme formation in wheat bran substrate was about 3500–4000 units/g. The culture of *Bacillus subtilis* PCSIR - 29, isolated after UV irradiation, was stable and, therfore, it was used for further studies.

Rate of Alpha-Amylase Production. Figure 1 shows the rate of alpha-amylase synthesis by *Bacillus subtilis* PCSIR - 29. The enzyme production was determined at different time intervals after inoculation. Maximum yield of the enzyme, i.e. 4000 unit/g wheat bran, was observed at 48 hr. Further, increase in fermentation time resulted in a decrease in enzyme formation. Thus optimum time for enzyme synthesis was 42–44 hr.

Effect of Partial Replacement of Wheat Bran on Fnzyme Formation. The data in Tables 5 and 6 show the synthesis of alpha-amylase when wheat bran was partially replaced by maize bran or rice bran. Of all the substrates examined,

Table 2. Effect of UV irradiation for different time periods on the cells of *Bacillus subtilis*, PCSIR - 2 for the synthesis of alpha-amylase in wheat bran by surface culture method.

Culture No.	Time for irradiation (min)	Wheat bran (units/g)
PCSIR - 2		370
1	20	1325
2	"	1350
3	"	1500
4	"	1500
5	"	1850
6	"	1425
7	"	1700
8	"	1475
9	"	1050
10	"	1400
11	30	1900
12	"	1600
13	"	1650
14	"	1500
15	"	1800
16	"	1500
17	"	1500
18	"	1700
19	40	2100
20	"	1700
21	"	1700
22	"	1400
23	"	1400
24	"	1200
25	"	1880
26	"	2300
27	"	3500
28	"	3250
29	"	4000
30	60	1200
31	"	2000
32	"	1600
33	"	1850
34	"	1575
35	"	1725
36	"	2050
37	"	2100
38	"	2275
39	"	1270
40	"	1390

wheat bran was found to be best for the production of alpha-amylase. Maize bran was better than rice bran, since no enzyme formation was found when wheat bran was completely replaced by rice husk. The amount of enzyme produced in maize bran, however, was 2500 unit/g maize bran.

Table 3. Effect of different concentration of 6 mercaptopurine on the cells of *Bacillus subtilis* PCSIR - 29 for alpha-amylase synthesis in wheat bran substrate.

No.	Concn on mutagen (mg/ml)	Time (hr)	Wheat bran (units/g)
PCSIR - 29	–	–	4000
1	0.100	5	2500
2			1500
3			2800
4			2625
5			2375
6	0.500	5	2920
7			2175
8			1730
9			1680
10			1500
11	1.50	5	2070
12			2350
13			1825
14			2480
15	0.100	20	2950
16			3100
17			3475
18			3850
19			3525
20			5850

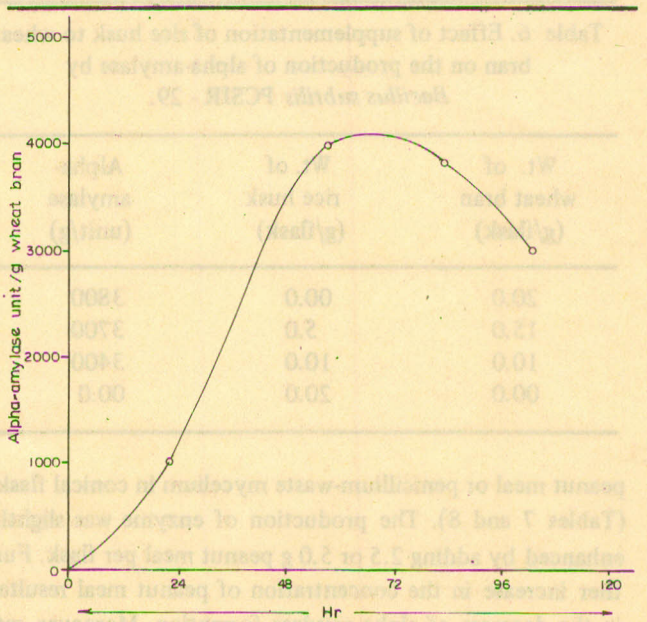

Fig. 1. Rate of alpha-amylase synthesis by *Bacillus subtilis* (PCSIR-29).

Effect of Different Nitrogen Sources on the Production of Alpha-Amylase. The biosynthesis of alpha-amylase was also investigated after partially replacing wheat bran by

Table 4. Effect of chemical treatment and UV irradiation alternatively on the the cells of *Bacillus subtilis* PCSIR - 20 for the synthesis of alpha-amylase in wheat bran substrate.

No.	Treatment			Wheat bran (unit/g)
	Chemical (mg/ml)	Time (hr)	Time for UV (min)	
PCSIR - 20	–	–	–	5850
1	0.100	20	30 min	4550
2				4775
3				4950
4				4850
5				5800
6				6300

Table 5. Effect of partial replacement of wheat bran by maize bran on the production of alpha-amylase by *Bacillus subtilis* PCSIR-29.

Wt. of wheat bran (g/flask)	Wt. of maize bran (g/flask)	Alpha-amylase (units/g)
20.0	0.0	4000
15.0	5.0	3700
10.0	10.0	3000
0.0	20.0	2500

Table 6. Effect of supplementation of rice husk to wheat bran on the production of alpha-amylase by *Bacillus subtilis* PCSIR - 29.

Wt. of wheat bran (g/flask)	Wt. of rice husk (g/flask)	Alpha-amylase (unit/g)
20.0	00.0	3800
15.0	5.0	3700
10.0	10.0	3400
00.0	20.0	00.0

peanut meal or penicillium-waste mycelium in conical flasks (Tables 7 and 8). The production of enzyme was slightly enhanced by adding 2.5 or 5.0 g peanut meal per flask. Further increase in the concentration of peanut meal resulted in the decrease of alpha-amylase formation. Moreover, rate of enzyme synthesis decreased with increasing penicillium-waste mycelium in the wheat bran cultures. Alpha-amylase production was 70% reduced when half of the wheat bran was replaced by the myelium.

The effect of the different nitrogen sources such as

Table 7. Effect of the addition of peanut meal to wheat bran substrate on the production of alpha-amylase by *Bacillus subtilis.*

Wt. of wheat bran (g/flask)	Wt. of peanut meal (g/flask)	Alpha-amylase (units/g)
20.0	00.0	3800
17.5	2.5	4200
15.0	5.0	4000
12.5	7.5	3700
10.0	10.0	2500

Table 8. Effect of the addition of penicillium-waste mycelium to wheat bran substrate on the production of alpha-amylase by *Bacillus subtilis.*

Wt. of wheat bran (g/flask)	Wt. of penicillium-waste mycelium (g/flask)	Alpha-amylase (units/g)
20.0	0.0	3800
17.5	2.5	2600
15.0	5.0	3200
12.5	7.5	2400
10.0	10.0	800

Table 9. Effect of urea and other inorganic nitrogen sources on the production of alpha-amylase by *Bacillus subtilis.*

Nitrogen source added	Alpha-amylase (units/g)
$NaNO_3$	3600
NH_4NO_3	3800
$(NH_4)_2SO_4$	3900
Urea	4200
Control	4000

$NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$ or urea (1 g N/l diluent) on alpha-amylase synthesis, was also studied (Table 9).

The production of enzyme increased slightly in the presence of urea only. Other nitrogen salts slightly decreased the enzyme synthesis.

DISCUSSION

Biosynthesis of alpha-amylase by the locally isolated culture of *Bacillus subtilis* was greatly improved by exposing the bacterial cells to UV radiation. The parent cultures

of *Bacillus species* produced 370 units/g wheat bran and, after mutation using UV radiations, the amount of enzyme synthesized was about 4000 units/g of wheat bran. The culture was further improved by exposing the cells to 6-mercaptopurine and UV radiations alternately but the culture isolated was found unstable. All the studies, therefore, were carried out using stable culture of *Bacillus subtilis* PCSIR - 29. Maize bran and rice husk were also evaluated as substrate for enzyme formation by solid substrate fermentation. Of all the substrates examined, however, wheat bran was found to be the best providing all nutrients for alpha-amylase synthesis. The addition of penicillium waste mycelium or peanut meal to the wheat bran culture medium was also investigated. Alpha-amylase formation was significantly affected when half of the wheat bran was replaced by peanut meal, of penicillin waste mycelium reduced engyme fromation. The mechanism of the inhibitory action of mycelium or peanut meal is not clear. The effect of different inorganic nitrogen sources or urea on enzyme synthesis was also studied. The addition of urea led to a slight increase in enzyme formation, the other sources of nitrogen showed on stimulatory effect.

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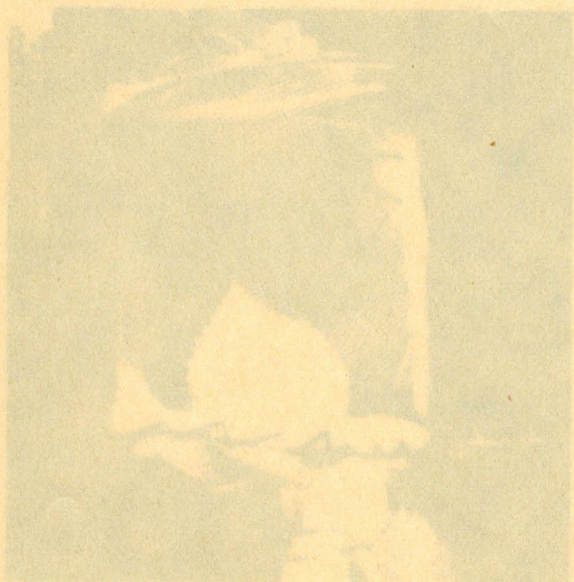

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