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Effect of Oilseed Cakes on α-Amylase Production by *Bacillus licheniformis* CUMC305

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The effects of oilseed cakes on extracellular thermostable α-amylase production by *Bacillus licheniformis* CUMC305 was investigated. Each oilseed cake was made of groundnut, mustard, sesame, linseed, coconut copra, madhuca, or cotton. α-Amylase production was considerably improved in all instances and varied with the oilseed cake concentration in basal medium containing peptone and beef extract. Maximum increases were effected by a low concentration (0.5 to 1.0%) of groundnut or coconut, a high concentration (3%) of linseed or mustard, and an intermediate concentration (2%) of cotton, madhuca, or sesame. The oilseed cakes made of groundnut or mustard could completely replace the conventional peptone-beef extract medium as the fermentation base for the production of α-amylase by *B. licheniformis*. The addition of corn steep liquor to cotton, linseed, sesame, or madhuca cake in the medium improved α-amylase production.

The thermostable α-amylases from *Bacillus* spp. play important roles in textile and paper industries, starch liquefaction, food, adhesive, and sugar production, and various other industries and therefore are of considerable commercial interest (9). It was reported earlier from this laboratory that the α-amylase of *Bacillus licheniformis* CUMC305 shows great promise because of its excellent activity at high temperatures (optimum, 91°C [retaining high activity even at 110°C]) and over a wide pH range (pH 5 to 10.0) (23), in contrast to the narrow pH range for other α-amylases (15, 24, 31, 32). In our previous studies of α-amylase production by this strain as well as studies of α-amylase production by other organisms, peptone-beef extract media were primarily used. Several studies have been undertaken to define ideal culturing and nutritional conditions for obtaining higher yields of the enzyme (11, 36), and various carbohydrate sources have been tested as enzyme production-increasing agents for different strains (19). Complex fermentation media which give optimum production of α-amylase have been reported (10), but no successful attempt to develop cheap substrates for optimum production of the enzyme by *B. licheniformis* has been reported. The regular use of peptone-beef extract-based fermentation media is not commercially viable for industries. For efficient commercial production, a continuous effort is being made to find cheaper substrate sources. Available carbon and nitrogen sources are the decisive factors in the optimum production of enzymes, and these differ very much from substrate to substrate. Defatted or whole vegetable meals like sorghum, wheat bran, cottonseed meal, soybean meal, and alfalfa meal are the most commonly used fermentation additives if not the principal substrates (29). Several oilseed cakes, because of their abundant availability and low price, are used as cattle feed (26), fertilizer (33), and, in rare cases after proper processing, food for humans (28). The use of complex starchy substances to achieve higher yields of α-amylase has been reported (3, 27). Baked-bean waste (12) has also been used for accelerating α-amylase production by fungi. The possibility that cheap raw materials like the various oilseed cakes increase the yield of thermostable α-amylases has not been much demonstrated. The present study was planned to determine the effect of oilseed cakes on enzyme production by the thoroughly studied organism *B. licheniformis* CUMC305 (4). In this paper, we report the enhancement of α-amylase production by most of the oilseed cakes tested. We also attempted to grow *B. licheniformis* CUMC305 in a medium containing some essential minerals and a few oilseed cakes as the sole carbon and nitrogen sources, excluding expensive bacteriological peptone and beef extract from the growth medium.

MATERIALS AND METHODS

Organism. The *B. licheniformis* CUMC305 isolate described earlier (23) was used throughout the study.

Fermentation media. Medium 1 had the following composition: 0.2% $(NH_4)_2HPO_4$, 0.05% Na_2CO_3, 0.02% K_2SO_4, 0.005% $MnCl_2 \cdot 4H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% starch (soluble), 0.2% beef ex-

TABLE 1. Effect of oilseed cakes on α-amylase production by *B. licheniformis* CUMC305

Concn of oilseed cake (%)	Saccharolytic α-amylase activity (U/ml)[a]						
	Groundnut	Coconut copra	Sesame	Madhuca	Cotton	Mustard	Linseed
0.5	8.69 (145)	6.49 (108)	6.99 (117)	8.12 (135)	8.59 (143)	7.99 (133)	1.99 (30)
1.0	8.69 (145)	6.54 (109)	8.12 (135)	8.37 (139)	8.72 (145)	8.69 (145)	3.5 (58)
1.5	5.85 (98)	6.54 (109)	8.74 (146)	8.37 (139)	9.78 (163)	8.69 (145)	4.74 (79)
2.0	5.23 (87)	4.98 (83)	9.75 (163)	8.74 (146)	10.5 (175)	11.54 (192)	7.12 (119)
2.5	5.23 (87)	2.98 (50)	9.5 (158)	8.5 (142)	10.3 (173)	11.54 (192)	9.49 (158)
3.0	5.23 (87)	2.15 (36)	9.5 (158)	8.5 (142)	10.09 (168)	11.54 (192)	9.49 (158)

[a] Control saccharolytic α-amylase activity, 6 U/ml (100%). Values in parentheses are percentages.

tract, 0.5% peptone (bacteriological), and 0.4% corn steep liquor. This medium was supplemented with six concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% [wt/vol]) of each oilseed cake tested. The pH was adjusted to 6.7. The following defatted oilseed cakes were obtained from markets: groundnut or peanut (*Arachis hypogaea*), mustard (*Brassica campestris*), sesame (*Sesamum indicum*), linseed (*Linum usitatissimum*), coconut copra (*Cocos nucifera*), madhuca (*Madhuca longifolia*), and cotton (*Gossypium hirsutum* r. *latifolium*).

Medium 2 contained 0.2% soluble starch and the mineral salts in medium 1. Oilseed cakes were added at concentrations of 1.5 and 2.0%. Corn steep liquor (0.1 or 0.4%) was added as required. After the pH values were adjusted to 6.7, 25-ml portions of media were placed in Erlenmeyer flasks, and sterilized at 15 lb/in^2 for 20 min.

Inoculum preparation. Cultures were maintained on nutrient agar slants with 0.2% starch. A loopful of the organism taken from a fresh slant was aseptically transferred to a 50-ml portion of medium 1, which was incubated at 49°C for 16 h. Cells were harvested by centrifugation, washed, and suspended in sterile distilled water. A 0.2-ml portion of this suspension (5.6×10^5 cells) was used as the inoculum.

Culture conditions and collection of enzyme. The inoculated flasks were incubated for 24 h at 49°C without shaking because shaking was found to be unfavorable for enzyme production. The culture filtrate was then tested for enzyme yield after centrifugation at 8,000 rpm for 10 min. The supernatant was collected and retained for assay of α-amylase activity in each case.

Assay of α-amylase. The method for assaying saccharolytic activity was based on the measurement of reducing power increase in a soluble-starch solution by the method of Sumner as modified by Bernfeld (2). The reaction mixture contained 0.5 ml of soluble starch (1.0% [wt/vol]), 0.3 ml of glycine-sodium hydroxide buffer (0.05 M; pH 9.0), and 0.2 ml of the enzyme solution. After 5 min of incubation at 90°C, 1 ml of 3,5-dinitrosalicyclic acid (DNS) reagent was added to stop the reaction. We prepared the reagent by dissolving 1 g of DNS in 20 ml of NaOH (final concentration, 2 N) and 30 g of sodium potassium tartrate in 50 ml of water, mixing the two solutions, and bringing the volume to 100 ml. The control contained the same quantities of soluble starch and buffer in the reaction mixture, but the enzyme solution (0.2 ml) was added after 1 ml of DNS reagent had been added to ensure complete inhibition of enzyme activity. The reagent blank contained 1 ml of distilled water and 1 ml of DNS reagent. We developed the color by holding all of the assay tubes in a boiling water bath for 5 min. The contents of each tube were then diluted with 10 ml of distilled water, and readings were taken in a Klett-Summerson colorimeter fitted with filter no. 54. The background DNS value was subtracted from the total DNS value after incubation with starch. One saccharolytic unit of α-amylase activity was calculated as the amount of enzyme which produced 1 mg of reducing sugar (as maltose) in 5 min.

Assays were also conducted by the method of Saito (31, 32), and it was noted that when assays were performed at 90°C for 10 min, one saccharolytic unit of α-amylase activity as described above was equivalent to 30 saccharolytic units of α-amylase activity (dex-

TABLE 2. α-Amylase production by *B. licheniformis* CUMC305 in media with oilseed cakes as the principal nutrient sources

Concn (%) of:		Saccharolytic α-amylase activity (U/ml)[a]						
Oilseed cake	Corn steep liquor	Groundnut	Coconut copra	Sesame	Madhuca	Cotton	Mustard	Linseed
1.5		7.93 (132)	2.71 (45)	5.91 (98)	1.52 (25)	4.69 (78)	6.74 (112)	4.83 (81)
1.5	0.1	7.83 (131)	2.17 (36)	7.39 (123)	6.96 (116)	6.1 (102)	6.63 (111)	6.3 (105)
1.5	0.4	8.23 (137)	3.24 (53.8)	7.5 (125)	4.8 (80)	6.8 (113)	7.21 (120)	7.5 (125)
2.0		8.15 (136)	4.78 (80)	7.82 (130)	1.3 (22)	5.11 (85)	6.08 (101)	4.78 (80)
2.0	0.1	7.717 (129)	3.33 (55)	8.36 (139)	2.06 (34)	6.6 (110)	7.717 (129)	6.52 (109)
2.0	0.4	8.53 (142)	2.99 (50)	7.72 (129)	4.6 (77)	7.02 (117)	7.82 (130)	7.83 (130)

[a] Control saccharolytic α-amylase activity, 6 U/ml (100%). Values in parentheses are percentages.

TABLE 3. Relevant chemical constituents of oilseed cakes

Oilseed cake	Protein (%)	Carbohydrate (%)	Major carbohydrate component(s)	Biotin (mg/lb)[a]	Pantothenic acid (mg/lb)	Thiamine (mg/lb)	Cystine (%)	Arginine (%)	Phenylalanine (%)	Isoleucine (%)	Reference(s)
Groundnut	51.8	26.9	Starch	—[b]	24.1	3.3	1.9	10.4	4.6	—	21, 25, 34, 35
Coconut copra	14.3	32.8	Sucrose	—	3.2	0.3	1.8	7.0	5.2	—	6, 17, 20, 21, 25
Sesame	36.0	14.7	Galactose, sucrose, glucose	0.027	4.3	1.09	2.7	29.2	10.8	10.5	13, 16, 18, 30
Madhuca	17.4	54.6	Glucose, rhamnose, arabinose, xylose	—	—	—	—	—	—	—	5, 7
Cotton	36.3	35.7	Sucrose, raffinose	0.13	11.0	3.2	2.0	7.4	6.8	3.4	1
Mustard	36.0	32.8	Starch, glucose	—	—	—	—	—	—	—	14, 34, 35
Linseed	30.5	43.2	Cellulose, sucrose, raffinose, xylose, galactose, rhamnose, arabinose	—	7.8	4.1	—	16.0	10.6	7.6	21, 22, 25, 35

[a] 1 lb = 453.592 g.
[b] —, Data not available.

trinizing power) as described by Saito, but when assays were conducted at 50°C for 10 min with α-amylase of *B. licheniformis* CUMC305, one saccharolytic unit of α-amylase activity as described above was only equivalent to 15 saccharolytic units (dextrinizing power) as described by Saito.

RESULTS

The oilseed cakes remarkably enhanced production of thermostable extracellular α-amylase by *B. licheniformis* CUMC305. The degree of enhancement, however, was relative to the concentration of oilseed cake (Table 1). We compared the effects of different concentrations of each oilseed cake with the results of a control experiment in which *B. licheniformis* CUMC305 was grown in conventional medium without any oilseed cake. The saccharolytic α-amylase activity in the control experiment was 6 U/ml, which was considered as 100%, and the relative enhancement of enzyme production was calculated accordingly.

It was quite evident that the groundnut seed cake had an additive effect at low concentrations only (0.5 or 1.0%) and an adverse effect at concentrations above 1.5% (Table 1). Defatted pressed coconut copra was found to be ineffective at low concentrations and retarding at concentrations above 1.5%. The best effect was obtained with mustard seed cake, which caused an increase in enzyme production of almost twofold at concentrations above 2%. Cottonseed cakes showed the next best effect, as determined by the direct correlation of the concentration gradient with the enzyme yield. Sesame and madhuca seed cakes had good effects at 2%, although only a slight decline from the peak was observed at concentrations of ≥2.5%. Linseed oil cakes improved α-amylase production only at 2% or higher.

Table 2 shows the effect of defatted oilseed crushings on α-amylase production in growth medium lacking peptone and beef extract. It is clear that groundnut and mustard seed cakes at each concentration could completely replace peptone and beef extract, and corn steep liquor at 0.4% further improved enzyme production. On the other hand, medium with sesame cakes at 2% was superior to control medium. However, the addition of 0.1% corn steep liquor to medium containing the sesame cake at even 1.5% resulted in at least 23% more enzyme.

The madhuca, cotton and linseed cakes were found to be insufficient by themselves as a nutrient source, but when used at 1.5% and supplemented with 0.1% corn steep liquor, they could effectively support growth and α-amylase production. Coconut copra, however, performed poorly, even in the presence of corn steep liquor.

DISCUSSION

It is evident from this study that oilseed cakes may serve as ideal fermentation bases for obtaining high yields of α-amylase from *B. licheniformis* CUMC305. Of the different oilseed cakes used as additives in peptone-beef extract medium, mustard cakes were the best, giving a twofold increase in enzyme production. Cottonseed cakes were the next best addition, giving a 1.75-fold increase. Groundnut, sesame, madhuca, and linseed gave 1.5- to 1.6-fold increases. Coconut copra, on the other hand, had a bad effect on enzyme production. In the absence of peptone and beef extract, defatted groundnut seed cake supplemented with corn steep liquor gave the highest enzyme yield of any of the oilseed cakes used. Groundnut cake has a well-balanced composition of nutrients essential for α-amylase production by *B. licheniformis* CUMC305 (Table 3; 4). Mustard oilseed cake contains a lower level of proteinaceous matter and a higher level of carbohydrate than does groundnut but is nevertheless a suitable nutrient source by itself for α-amylase production. Therefore, it is assumed that the carbohydrates of this oilseed cake are very suitable for α-amylase production enhancement.

Sesame oilseed cake contains all of the vitamins and excessive amounts of four of the amino acids essential for enzyme production by *B. licheniformis* CUMC305. It is quite likely that the concentrations of the essential nutrients are not found in a well-balanced ratio, thus leading to reduced enzyme production. The improvement in α-amylase production by supplementation of the medium with 0.1% corn steep liquor suggests that corn steep liquor contains an essential growth factor or nutrients in a well-formulated proportion for higher α-amylase production.

Madhuca oilseed cake is a poor protein source with a fairly good amount of essential carbohydrate. When medium 2 containing madhuca oilseed cake at 1.5% was supplemented with a nitrogenous growth factor like 0.1% corn steep liquor, enzyme production increased almost five times over the production obtained when 1.5% oilseed cake was used alone.

The linseed and cotton oilseed cakes were insufficient by themselves to enhance α-amylase production, and corn steep liquor was required to provide the rest of the nutrients essential for growth and enzyme production. Linseed and madhuca are rich in carbohydrates but, possibly because of a deficiency of some amino acids, are poor substrates for supporting high enzyme yields. Coconut copra oilseed cake proved to be of low nutritive value, in comparison with all of the other oilseed cakes studied.

The study of the effect of oilseed cakes on α-amylase production by *B. licheniformis* is of interest because the cakes can completely replace peptone and beef extract, which are costly and impractical for the commercial production of the enzyme. The oilseed cakes, by-products of oil industries, are very inexpensive, readily available, natural, and fairly well-balanced nutrient sources.

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