Pakistan J. Sci. Ind. Res., Vol. 18, Nos. 1–2, February–April 1975

PREPARATION OF AMYLOGLUCOSIDASE BY SOLID SUBSTRATE FERMENTATION

M. A. Qadeer and J. I. Anjum

PSCIR Laboratories, Lahore 16

(Received April 2, 1974; revised December 14, 1974)

Abstract. Brans of various cereals such as wheat, maize and gram, and rice husk were evaluated as substrate for mould growth for the biosynthesis of amyloglucosidase by the strains of *Aspergillus* and *Rhizopus* species. The culture extracts of all mould strains, when grown on various substrates, possessed amylogulcosidase activity of 1900–2200 units/g bran, 48 hr after spore inoculation. The use of wheat bran, however, was extensively studied for enzyme production. The optimum cultural conditions such as incubation time, thickness of bran layer, various diluents and their ratios to wheat bran and method of cooking (autoclaving and steaming) were determined. The use of spent wheat bran resulted in the increase of enzymic activity by both mould strains. The supplementation of wheat bran with various sources of carbohydrates or nitrogen including penicillin-waste mycelium did not improve the enzyme synthesis.

In continuation of our studies as reported earlier[1] on the biosynthesis of amyloglucosidase by *Aspergillus* species in shake flasks, the present work describes the enzyme production by surface culture process using wheat bran or brans of other cereals, and rice husk as substrate for mould growth. This type of fermentation process is quite feasible in an agricultural country like Pakistan, where brans of all cereals are available in large amount for their commercial utilization. The surface culture process or Koji process, very commonly used in Japan, requires a large surface area for trays but other factors such as rates of agitation and aeration, foaming, contaminations, or pH control are not serious problems as in fermentations in deep tanks. Moreover, a considerable amount of work on the production of fungal amylase has been carried out by the surface culture process, using wheat bran as a substrate.[2–11] A few reports are available in the literature describing the production of amyloglucosidase by growing mould on wheat bran,[12–14] most of the work, otherwise, has been carried out by submerged fermentation process.[15–18]

Attempts were made to explore the possibilities for the exploitation of bran of various cereals or wheat bran in particular, for enzyme production in conical flasks before pilot-plant studies. The factors studied are selection of mould strains, thickness of bran layer, selection of diluents and its ratio to wheat bran and supplementation of wheat bran by various sources of carbohydrates or nitrogen including penicillin-waste mycelium.

Materials and Methods

Organism. The mould strains of *Aspergillus awamorie* NRRL 3112; *A. phoenicis* IFO 6649, IFO 6650 and UAM-10; *A. oryzae* IFO 4290 and IMI 17299 and *Rhizopus niveous* IFO 4759 were used in the present investigation. The cultures were maintained on the agar medium consisting of (g/1);sucrose 30.0, $NaNO_3$ 3.0, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, agar 15.0. The cultures were incubated at 30±2°C for 5–7 days for maximum sporulation and then slants were kept in the refrigerator.

Inoculum Preparation. The spores from 5–7 day-old cultures were wet with 5 ml 0.05% Monoxal O.T. (diacetyl ester of sodium sulphosuccinic acid). The supernatant-containing-spores was decanted off asceptically. The agar surface was washed twice with sterile distilled water. The combined washings were made up to 25 ml and shaken with glass beads to break the clumps of spores.

Fermentation Procedures. Wheat bran, gram bran and rice husk were purchased from the local market and maize bran was obtained from Glaxo Laboratoreis, Lahore. Cereal bran or rice husk (20 g) in a litre cotton-plugged conical flask was moistened with 30 ml diluent and autoclaved at 121°C for 20 min. The cooled bran was inoculated with 1 ml spore suspension and incubated at 30±2°C for 48 hr. The cultures were daily shaken during mould growth.

Preparation of Enzyme Extract. A ratio of 10 parts of phosphate buffer, KH_2PO_4, 3.5 g/1 and KH_2PO_4 1.5 g/1 of pH 6.5 to one part by weight of original dry bran was used for extraction. The cake of mould bran was shaken on a rotary shaker for 1 hr. at room temperature. To this mould bran suspension was added 1 ml of a 20% $CaCl_2.2H_2O$ solution for each 40 ml phosphate buffer and the culture was shaken for another 15 min. The precipitate and bran were separated by centrifuging, yielding a clear enzyme extract.

Analytical Method. Amyloglucosidase activity of the mould bran extract was determined by the method described in the Technical Bulletin 2–112 of Miles Laboratories. One unit of amyloglucosidase enzyme was taken as that amount of enzyme required to catalyse under assay condition the production of 1 g glucose from strach in 1 hr at 60°C.

Results

Strain Selection. The amyloglucosidase activity of the mould bran extract of various *Aspergillus*

TABLE 1. COMPARISON OF THE RATE OF AMYLOGLUCOSIDASE SYNTHESIS BY DIFFERENT MOULD STRAINS IN WHEAT BRAN.

Organism	Amyloglucosidase (μ/g)		
	48 hr	72 hr	120 hr
A. awamorie NRRL-3112	1941	2023	2030
A. phoenicis IFO 6649	2169	2060	2060
A. phoenicis IFO 6650	2069	2084	2060
A. phoenicis UAM-10	1969	2060	2084
A. oryzae IFO 4290	1934	2040	2131
A. oryzae IMI 17299	2080	2076	2084
Rhizopus niveous IFO 4759	1949	2054	2084

TABLE 2. EFFECT OF VARIOUS DILUENTS ON THE BIOSYNTHESIS OF AMYLOGLUCOSIDASE BY *A. phoenicis* IFO 6649 AND *R. niveous* IFO 4759 (INCUBATION TIME 48 hr).

Mash diluent	Amyloglucosidase (μ/g)	
	A. phoenicis IFO 6649	*R. niveous* IFO 4759
Water	1975	1900
Phosphate buffer	2100	1971
HCl (0.1N)	2200	2050
DAP	2088	1904

and *Rhizopus* species are given in Table 1. Phosphate buffer was used as diluent. The enzymic activity was assayed 48, 72 and 96 hr after spore inoculation. Of all the mould strains, *A. phoenicis* IFO 6649 gave slightly higher enzymic activity, i.e. 2169 units/g 48 hr after spore inoculation. Further increase in the incubation time did not increase significantly the amyloglucosidase activity. The optimum time of 48 hr, therefore, was selected for culture growth. *Aspergillus* species in general also produce transglucosidase which forms isomaltose and other oligosaccharides, these sugars interfere in the crystallization of glucose. The mould bran extracts of *Rhizopus* species, however, were usually free from transglucosidase. *A. phoenicis* IFO 6650 and *R. niveous* IFO 4759 were, therefore, selected for the determinatiin of their optimum cultural conditions.

Selection of Diluent. Bran mashes were prepared with 1 part of bran to 1.5 parts of diluents such as tap-water, phosphate buffer, 0.1N HCl and 0.3% diammonium phosphate (Table 2). Amyloglucosidase activity was slightly greater in the presenc- of HCl or phoshate buffer by both fungal strains. The use of HCl as mash diluent, however, was preferred as the chances for contaminants to grow are greatly minimized at such a low pH. Moreover, the use of HCl on an industrial scale, as mash diluent would be more economical than phosphate buffer.

Sterilization of Bran. Wheat bran containing 0.1N HCl as diluent in conical flasks was steamed for different periods ranging from 15 to 150 min before

TABLE 3. EFFECT OF TIME OF STEAMING WHEAT BRAN ON AMYLOGLUCOSIDASE ACTIVITY OF *A. phoenicis* IFO 6649 AND *R. niveous* IFO 4759 (INCUBATION TIME 48 hr).

Steaming time (min)	Amyloglucosidase (μ/g)	
	A. phoenicis IFO 6649	*R. niveous* IFO 4759
0	2000	2025
15	2068	1950
30	2088	2010
60	2100	2101
90	2100	2056
120	2100	2100
150	2082	2023
Sterilization 15	2045	2078

TABLE 4. AMYLOGLUCOSIDASE ACTIVITY OF EXTRACTS OF CULTURES OF *A. phoenicis* IFO 6649 AND *R. niveous* IFO 4759 GROWN ON WHEAT BRAN AND RELATED SUBSTRATES.

Substrate	Amyloglucosidase (μ/g)	
	A. phoenicis IFO 6649	*R. niveous* IFO 4759
Wheat bran	2065	2049
Maize bran	2180	2147
Gram bran	2280	2280
Rice husk	2060	2088
Wheat bran+maize bran*	2060	2060
Wheat bran+rice husk*	2078	2060
Maize+gram bran*	2072	2040
Maize+rice husk*	2060	2059
Gram bran+rice husk*	2078	2040
Spent wheat bran	2322	2540

* Equal parts by weight of the two components.

inoculation. The bran mash cultures were also run parallel which were inoculated after autoclaving at 15 lb pressure for 30 min (Table 3). The enzymic activity of all the cultures in which wheat bran was inoculated after steam-sterilization, was about the same as that of wheat bran inoculated after autoclaving. The low pH of the bran mashes is of great significance in controlling the activity of contaminants. Thus steaming of the wheat bran mashes for 60 min instead of autoclaving at higher pressure is quite effective for mould growth and for the synthesis of amyloglucosidase.

Selection of Substrate. The data of Table 4 shows amyloglucosidase activity of *A. phoenicis* and *R. niveous* when the mould strains were grown on various brans such as wheat, maize, gram and rice husk. The enzymic activity was greater when the cultures were grown on maize bran, gram bran or rice husk, than that on wheat bran. Amyloglucosidase synthesis was also better when the mould strains

TABLE 5. EFFECT OF DEPTH OF BRAN LAYER ON THE AMYLOGLUCOSIDASE ACTIVITY OF *A. phoenicis* IFO 6649 AND *R. niveous* IFO 4759 (INCUBATION TIME 48 hr).

Wheat bran g/l flask	Depth cm	Amyloglucosidase (μ/g)	
		A. phoenicis IFO 6649	*R. niveous* IFO 4759
10	—	2060	2020
20	0.8	2080	1940
30	1.6	2120	2040
40	1.8	1980	2020
50	2.2	2060	2020
60	2.5	2060	2040
70	3.4	1950	1950
80	3.6	1940	1950
100	4.2	1900	1964
120	4.4	1913	1940

were grown on equal parts by weight of two components, i.e. wheat bran (10 g)+maize bran (10 g). The production of enzyme by both the strains was greatly increased when spent wheat bran was employed as substrate. This stimulatory effect may be due to the presence of proteinous matter of the mycelium in the spent wheat bran.

Effect of Depth of Bran Layer. In solid substrate fermentation, the depth of bran layer for culture growth is of great importance for its commercialization. During mould growth the temperature of the bran mashes increases rapidly, i.e. above 45°C. Such a rise in temperature, therefore, may result both in a decrease in amyloglucosidase activity and in mycelial growth. Thus, different amount of wheat bran such as 10–100 g/l flasks with 0.4–4.4 cm depth were used for mould growth and enzyme synthesis. Amyloglucosidase formation in flasks was little affected with an increase in the depth of bran layer. Since shaking of bran cultures was easier in flasks than in trays, the depth of 2.5 cm in trays would be feasible on a commercial scale.

Effect of Supplements. The data of Tables 6 and 7 indicate the effect of the addition of sugar or penicillin-waste mycelium to the bran cultures on the enzyme synthesis by mould strains respectively. Amyloglucosidase activity of all the culture remained unchanged as compared with the control culture. Thus enzyme formation was not induced by the addition of sugar or penicillin-waste mycelium as source of nitrogen.

Extraction of Enzyme. To extract the enzyme, the culture bran was mixed thoroughly with the required volume of water (1:10) and the flasks were shaken on a rotary shaker for 1 hr. Then clear filtrate was obtained by centrifuging in a basket centrifuge. Extracts obtained with 0.01M solutions of NaOH, $CaCl_2$, Na_2CO_3, NaCl, HCl, phosphate buffer and 0.1% wetting agents (Monoxal O.T.) and 0.1% Tween 80 possessed about the same enzyme activity as that obtained with water.

TABLE 6. EFFECT OF SUGARS ON THE BIOSYNTHESIS OF AMYLOGLUCOSIDASE IN WHEAT BRAN.

Sugar	Amyloglucosidase (μ/g) (sugar concn)			
	A. phoenicis IFO 6649		*R. niveous* IFO 4759	
	3.75 mg/g	7.5 mg/g	3.75 mg/g	7.5 mg/g
Lactose	1947	1910	2078	2118
Glucose	2009	2108	2075	2094
Maltose	2108	2018	2120	2104
Sucrose	2090	2000	2094	2094
Strach	2094	2100	2100	2100
Control	1984	—	1972	—

TABLE 7. EFFECT OF THE SUPPLEMENTATION OF PENICILLIN-WASTE MYCELIUM IN THE WHEAT BRAN SUBSTRATE ON THE AMYLOGLUCOSIDASE ACTIVITY (INCUBATION TIME 48 hr).

Penicillin-waste mycelium (%)	Amyloglucosidase (μ/g)	
	A. phoenicis IFO 6649	*R. niveous* IFO 4759
0.0	2180	2085
10.0	2100	2085
20.0	2100	2069
25.0	2092	2046

The use of phosphate buffer, however, was preferred since precipitation of phosphate as $Ca_3(PO_4)_2$ helped in the clarification of the culture filtrate.

Discussion

The present work shows that mould strains of *A. phoenicis* and *R. niveous* produce large amount of the enzyme amyloglucosidase and their use for pilot-plant production of the enzyme is quite feasible. Both the mould strains, particularly *A. phoenicis* displayed characteristics necessary for growing commercially due to its rapid growth that covers the bran thus greatly reducing the activity of contaminants. Wheat bran after steaming is ideal for mould growth and enzyme formation. The bran of other cereals, such as maize, gram or rice husks are equally good for their commercial exploitation in the manufacture of amyloglucosidase.

The important factors governing the enzyme formation are thickness of bran layer, initial pH of mash for controlling the activity of contaminants and method of extraction. The bran layer of 2.5 cm is the most suitable for maximum enzyme synthesis. Further increase in bran thickness will not be economical since mould grows on the upper layer of the bran. Moreover, decrease in bran layer thickness

would require more surface area thus making the process uneconomical. Incubation time of 48 hr was optimum for all mould strains, further increase in the growth period did not improve the enzyme formation significantly. Wheat bran provides all the nutrients necessary for the rapid mould growth. Amyloglucosidase formation remained unchanged when supplementation of various sugars or nitrogen to the wheat bran mash was made. The addition of penicillin-waste mycelium to the wheat bran mash also did not stimulate enzyme synthesis as reported earlier in shake flask experiments using synthetic strach–salts medium. The non-stimulatory effect of penicillin-waste mycelium on enzyme synthesis in wheat bran mash is obscure. It may be due to its effect on the porosity of wheat bran. The use of HCl as mash diluent resulted in a low pH and thus chances for contaminants to grow were greatly minimized. The enzyme formation, however, was greater when bran mash was supplemented by spent wheat bran. The stimulatory effect of spent wheat bran needs thorough studies. It may be due to the mycelial proteins present or the modified starch or cellulose components of the spent wheat bran. The supplementation of wheat bran mash with the spent wheat bran, however, makes the process more economically feasible.

Acknowledgement. The authors gratefully acknowledge the technical help of Messers. M. Salim, Mehboob Ali Qureshi, Muhammad Hameed and Noor Mohammad, throughout the investigations.

References

1. M.A. Qadeer and Kausar Tasnim, Pakistan J. Sci. Ind. Res., **14,** 247 (1971).
2. K. Arima, W. J. Nickerson, M. Pyke, H. Schandorl, A. C. Thaysen and R. S. W. Thorne, *Yeasts* (Dr. W. Junk, Prague, 1957),
3. Y.J. Takeda, Agr. Soc. Japan, 35, 845, 943 (1935).
4. J. Takamine, U.S. Patent, 525,820 (1894).
5. J. Takamine, U.S. Patent, 525,823 (1894).
6. J. Takamine, U.S. Patent, 991,560 (1911).
7. L.A. Underkofler, G.M. Severson and K.J. Geering, Ind. Eng. Chem., **38,** 980 (1946).
8. J. Takamine, Ind. Eng. Chem., **6,** 824 (1914).
9. L. B. Lockwood, Trans N. Y. Acad. Sci., Ser II, **15,** 2 (1952).
10. J. A. Shellenberger, Chem. Eng., **54,** 130 (1947).
11. J. Tagawa, L. Uchida, I. Iguchi, K. Shiraska and S. Kohayshi, Ann. Rept. Takamine Lab., **4,** 63 (1952).
12. H. Toguchi, T. Imanak, S. Teramato, M. Takatsu and M. Sato, J. Ferment. Technol., **46,** 823 (1968).
13. Fwu-Ching Lin, Chin-Chuan Chang and I-lung Wang, J. Chinese Agr. Chem. Soc., **7,** 31 (1969).
14. Fwu-Ching Lin, J. Chinese Agr. Chem. Soc., **7,** 25 (1969).
15. K.L. Smiley, M.C. Cadmus, D.E. Hensley and A.A. Lagod, Appl. Microbiol., **12,** 455 (1964).
16. D.R. Lineback, C.E. Georgi and R.L. Doty, J. Gen. Appl. Microbiol. (Tokyo), **12,** 27 (1966).
17. M. Okazaki and G. Terui, J. Ferment. Technol., **44,** 287 (1966).
18. L.L. Barton, D.R. Lineback and C.E. Georgi, J. Gen. Appl. Microbiol. (Tokyo), **15,** 327 (1969).