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Production of α-Amylase with *Aspergillus oryzae* on Spent Brewing Grain by Solid Substrate Fermentation

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Abstract

Ten *Aspergillus oryzae* strains were screened in solid substrate fermentation for α-amylase production on spent brewing grain (SBG) and on corn fiber. SBG proved to be a better substrate for enzyme production than corn fiber. A Plackett-Burman experimental design was used to optimize the medium composition for the best strain. Solid substrate fermentation on optimized medium with *A. oryzae* NRRL 1808 (=ATCC 12892) strain in stationary 500-mL Erlenmeyer flask culture yielded 4519 U of α-amylase/g of dry matter substrate in 3 d. The whole solid substrate fermentation material (crude enzyme, *in situ* enzyme) may be considered a cheap biocatalytic material for animal feed rations and for bioalcohol production from starchy materials.

Index Entries: Amylase; solid substrate fermentation; *Aspergillus oryzae*; spent brewing grain; Plackett-Burman experimental design.

Introduction

α-Amylase (α-1,4-glucanohydrolase [EC 3.2.1.1], endoamylase and dextrogenic) is of widespread occurrence in nature. The enzyme hydrolyzes α-1,4-glucosidic linkages in amylose, amylopectin, and glycogen in an endo-acting mechanism *(1)*. Amylases have a wide spectrum of industrial

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applications. Fungal amylases have been used to manufacture starches, starch derivatives, and starch saccharification products. The most important fungi used for α-amylase production are *Aspergillus oryzae*, *A. niger*, and *Rhizopus oryzae (2)*.

Solid substrate fermentation offers numerous advantages over submerged fermentation systems, including high volumetric productivity, high concentration of the products, less effluent generation, and simple fermentation equipment *(3,4)*. Fungal amylolytic enzymes are frequently produced by solid substrate fermentation *(5)*. For example, *koji* made from rice or barley is an important source of starch-degrading enzymes needed for sake brewing *(6)*.

Solid substrate fermentation may also be used for value addition to different agroindustrial residues *(7–9)*. Crude (*in situ*) hydrolytic enzymes prepared by solid substrate fermentation (the whole solid substrate fermentation culture) can be used in biotechnologic processes, such as ensiling, feed supplementation, and bioprocessing of crops and crop residues *(9)*. Among several such agroindustrial residues, which could be used as substrates, spent brewing grain (SBG) constitutes an interesting opportunity. About 650,000 t of dry SBG is produced annually in the United States alone. SBG is mainly the residual seed hull and fiber of malt and barley after the brewing process. Corn fiber (corn seed hull), a byproduct of the corn wet-milling industry, is another potential agroindustrial residue that could be used as substrate for bioprocesses. More than 4 million t/yr of corn fiber is produced in the United States *(10,11)*. Presently, the majority of SBG is used as cattle feed. SBG and corn fiber can be used for producing value-added products such as animal feed supplement and/or crude (*in situ*) enzyme by microbial fermentation. For example, SBG was used as a substrate for cellulase, xylanase *(9,12)*, α-amylase, and amyloglucosidase *(13)* production in solid substrate fermentation.

The productivity of any microbial process is largely affected by the process parameters and media composition. For medium optimization, various statistical experimental designs are widely used *(14)*. The Plackett-Burman design, one of the most frequently applied methods, was successfully adopted for medium optimization of α-amylase production by *Aspergillus flavus* on *Amaranthus* grains in solid substrate fermentation *(15)*.

The objectives of the present study were to compare 10 *A. oryzae* strains in solid substrate fermentation for α-amylase production on SBG and corn fiber, and to optimize the physical parameters and medium composition with the most productive strain on SBG.

Materials and Methods

Microorganisms and Culture Conditions

A. oryzae strains were obtained from the following culture collections: American Type Culture Collection (ATCC), Manassas, VA; International Mycological Institute, Egham, Surrey, UK (IMI); University of Sciences

Jozsef Attila, Szeged, Hungary (JATE); Northern Regional Research Laboratory (NRRL), USDA, Peoria, IL; Russian Culture Collection, Moscow (VKM).

The strains were maintained on potato dextrose agar (PDA) plates. The total viable spore number in PDA Petri plate culture (8 d old) was determined by counting colonies following serial dilution.

Substrates

SBGs were obtained from three different factories: Coors Brewery, Golden, CO; Dreher Brewery, Budapest, Hungary; New Belgium Brewery, Fort Collins, CO. The freshly collected (wet) samples were dried overnight at 80°C. The composition of the Coors SBG was (% of dry matter): hemicellulose (23); cellulose (15); starch (12); protein (18); lignin (22); other (10). Untreated corn fiber (corn seed hulls) was obtained from Pekin Energy, currently known as Williams Companies, Pekin, IL. The fiber was collected from the fiber dewatering presses and then dried overnight in force-draft ovens at 50°C.

Fermentation

Nonoptimized solid substrate fermentation was carried out in 500-mL cotton-plugged Erlenmeyer flasks. Five grams of dry substrate was supplemented with salt solution to adjust the moisture level to between 50 and 83%. The composition of the salt solution was as follows: 5 g/L of NH_4NO_3, 5 g/L of KH_2PO_4, 1 g/L of NaCl, 1 g/L of $MgSO_4 \cdot 7H_2O$, and 2 mg/L of $CoCl_2 \cdot 6H_2O$, 1.6 mg/L of $MnSO_4$, 3.4 mg/L of $ZnSO_4 \cdot 7H_2O$, 5 mg/L of $FeSO_4 \cdot 7H_2O$. In the time-course experiment, the following composition of optimized medium was used: 5 g of SBG supplemented to 67% moisture content with the following solution: 50 g/L of soluble starch (E. Merck, Darmstadt, Germany), 20 g/L of corn steep liquor (CSL) (Sigma), 20 g/L of soybean meal (Cereol, Hungary), 3 g/L of NH_4NO_3. The prepared substrate was sterilized at 121°C for 30 min. After cooling, the medium was inoculated with a spore suspension of the fungus to a final concentration of 2×10^7 spores/g of dry matter (DM). The spore suspension was prepared by washing spores from the surface of 8-d-old sporulating PDA plates with water containing 0.1% Tween-80. The inoculated flasks were incubated at 25°C for 5 d. All solid substrate fermentation experiments were carried out in duplicate in single experiments, and the results shown are average values.

Extraction

Enzyme activity was determined from the culture extract of solid substrate fermentation samples: 5 g dry wt of fermented substrate was extracted with 100 mL of water containing 0.1% Tween-80, by shaking for 1 h at room temperature (25°C). At the end of extraction, the suspension was centrifuged (3000*g*, 10 min). Supernatants were stored at 4°C until the assays were performed.

Table 1
Plackett-Burman Matrix for $N = 12$

	Variable										
Medium	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*
1	+	−	+	−	−	−	+	+	+	−	+
2	+	+	−	+	−	−	−	+	+	+	−
3	−	+	+	−	+	−	−	−	+	+	+
4	+	−	+	+	−	+	−	−	−	+	+
5	+	+	−	+	+	−	+	−	−	−	+
6	+	+	+	−	+	+	−	+	−	−	−
7	−	+	+	+	−	+	+	−	+	−	−
8	−	−	+	+	+	−	+	+	−	+	−
9	−	−	−	+	+	+	−	+	+	−	+
10	+	−	−	−	+	+	+	−	+	+	−
11	−	+	−	−	−	+	+	+	−	+	+
12	−	−	−	−	−	−	−	−	−	−	−

Enzyme Assay

α-Amylase activity was determined as described by Okolo et al. *(16)*. The reaction mixture consisted of 1.25 mL of 1% soluble starch (E. Merck) solution, 0.25 mL of 0.1 *M* sodium acetate buffer (pH 5.0), 0.25 mL of distilled water, and 0.25 mL of properly diluted crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalent) were estimated by the dinitrosalicylic acid method of Miller *(17)*. One unit of amylase is defined as the amount of enzyme releasing 1 μmol of glucose equivalent/min under the assay conditions.

Experimental Design

To optimize solid substrate fermentation medium, a Plackett-Burman experimental design was used. This fractional method allows the testing of multiple independent variables within a single experiment *(18)*. Experimental designs for 8, 12, 16, and up to 100 variables in multiples of four may be selected using series of arrays constructed for this purpose. Table 1 shows an array for $N = 12$ trials that will test $N - 1$ independent variables. Each row represents one trial (fermentation medium) and each column represents a single variable (medium component). The + and – elements represent the two different levels of each variable present within each trial. After the independent variables and their corresponding levels have been selected, the trials are performed and the responses, such as enzyme production, are measured. The effect of each variable on the measured response is determined by the difference between the average of the + and – responses (illustrated in the Pareto chart of effects in Fig. 1). Experimental error is estimated from the dummy variables and represents the degree of expected variability within the experiment. The significance level of the effect of each variable is determined by the student's *t*-test:

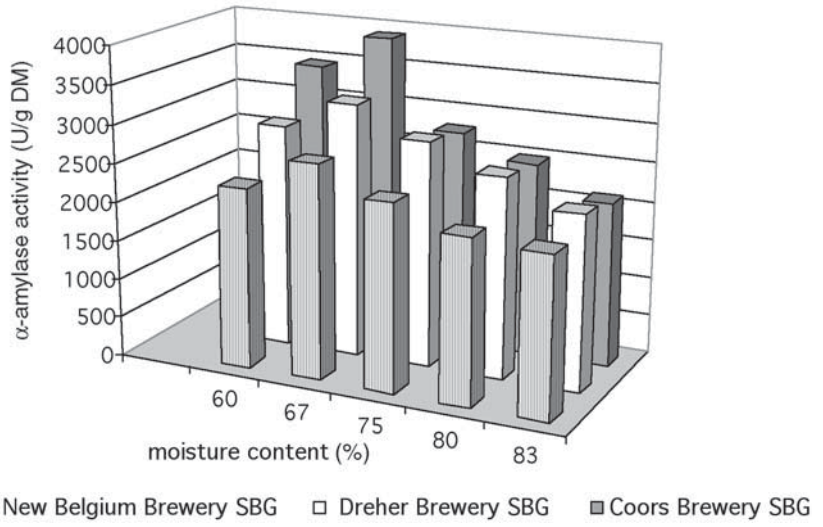

Fig. 1. Pareto chart of effects.

$$t(12) = \frac{b_j}{s_{b_j}}$$

in which b_j represents the regression coefficient, calculated as follows:

$$b_j = \frac{\sum_i y_i x_{ji}}{\sum_i x_{ji}^2}, \qquad b_0 = \frac{\sum_i y_i}{\sum_i x_{ji}^2}, \qquad \sum_i^N x_{ji}^2 = N$$

in which s_{b_j} is the standard error of the regression coefficient, y_i is the α-amylase activity, x_{ji} is the transformed factors, b_0 is the intercept, and N is the number of trials.

The effect of a factor is considered to be significant if $t_{\alpha/2} < t(12)$. $t_{\alpha/2} = 2.179$ when $\alpha = 0.05$ and degrees of freedom is 12.

Eleven medium ingredients—3 carbon sources, 3 organic nitrogen sources, 1 inorganic nitrogen source, and 4 minerals—were tested (Table 2). The data from the production of α-amylase were subjected to statistical analysis. The regression coefficients of the individual ingredients on the yield and the t values for the regression coefficients were determined. The ingredients having the highest t values were considered the best nutrients.

Results and Discussion

Of the ten *A. oryzae* strains examined, strain NRRL 1808 exhibited the highest α-amylase production on SBG. This strain was indicated as a good amylase producer in a mold bran process (www.atcc.org). Corn fiber was much inferior to SBG in enzyme production (Table 3).

Table 2
Ingredients Chosen
for Plackett-Burman Experimental Design

	Level (w/v%)	
Ingredient	–1	+1
Starch	1	5
Maltose	1	5
Glucose	1	5
CSL	0	2
Soybean meal	0	2
Canola meal	0	2
NH_4NO_3	0	0.3
NaCl	0	0.3
KH_2PO_4	0	0.3
$MgSO_4$	0	0.1
$CaCl_2$	0	0.1

Table 3
Comparison of α-Amylase Production by Different *A. oryzae* Strains
on Corn Fiber and SBG in Nonoptimized Fermentation[a]

	α-Amylase activity (U/g DM)	
A. oryzae strain	Corn fiber	SBG (Dreher)
ATCC 1011 (=NRRL 447)	261	1830
NRRL 451 (=ATCC 16868)	82	899
NRRL 1808 (=ATCC 12892)	128	2622
NRRL 1989 (=ATCC 14895)	15	106
NRRL 2217 (=ATCC 11493)	33	261
NRRL 3485 (=ATCC 46244)	181	1561
NRRL 6270 (=ATCC 46249)	134	1047
JATE 0451	94	373
IMI 17299	352	2258
VKM F-762	373	2198

[a]Fermentation conditions: 30°C, 75% moisture content, initial pH = 6.0, 5 d.

The optimization of physical parameters was carried out using the single-factor method by keeping other factors at unspecified constant levels.

The moisture content of the medium in solid substrate fermentation is very important for the growth of microorganisms, production of enzymes, and enzyme activity. The optimum moisture level must be determined for each system and microorganism *(19)*. To examine the effect of moisture content on α-amylase production, moisture levels of 50, 60, 67, 75, and 83% were tested on three different SBGs. Maximum α-amylase production was obtained at 67% moisture content for all three SBGs (Fig. 2). Coors SBG was superior to Dreher and New Belgium SBGs in enzyme production (Fig. 2).

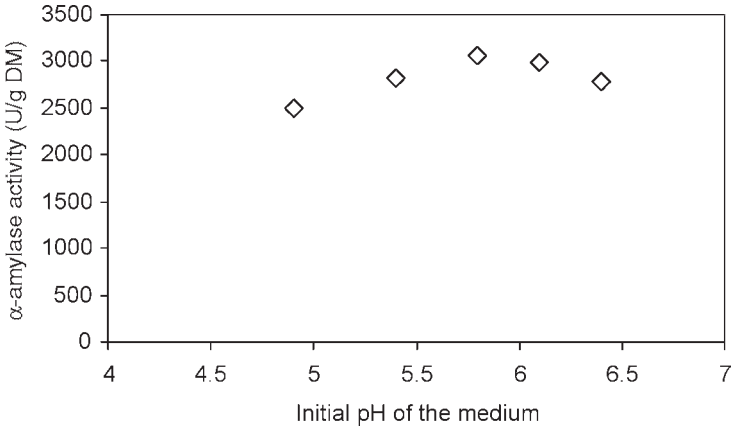

Fig. 2. Production of α-amylase by *A. oryzae* NRRL 1808 strain on SBGs from different breweries in nonoptimized media at different moisture levels. Fermentation conditions: 30°C, initial pH = 6.0, 5 d.

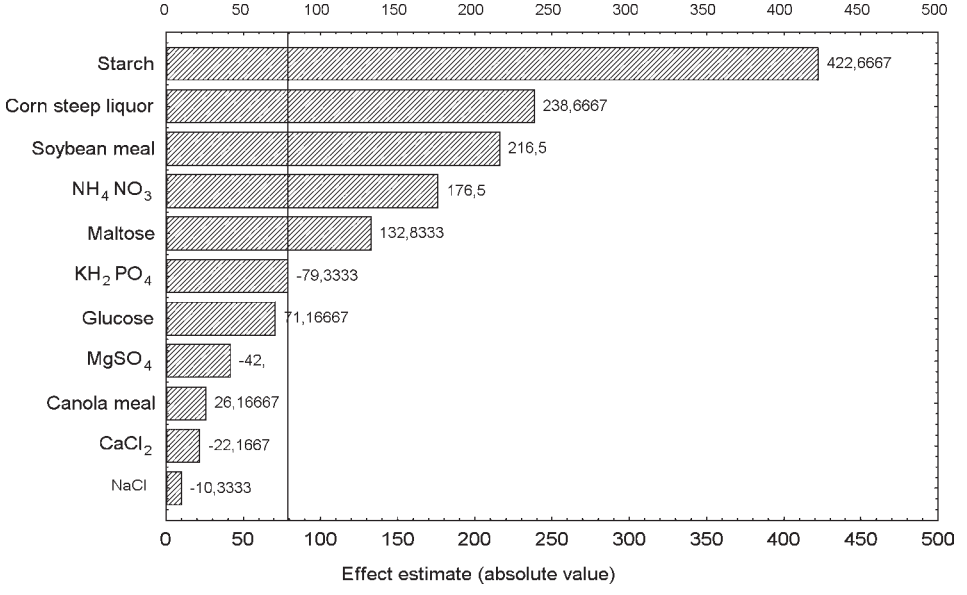

Fig. 3. Effect of initial pH of medium on α-amylase production of *A. oryzae* NRRL 1808 strain. Fermentation conditions: Dreher SBG, 67% moisture content, 25°C, 5 d.

To evaluate the effect of incubation temperature on α-amylase production, solid substrate fermentation was carried out at three different fermentation temperatures: 25, 30, and 37°C. The highest α-amylase yield was obtained at 25°C (data not shown). Studies on the initial pH of the medium were conducted. The pH only slightly influenced the α-amylase yield. The optimum pH was approx 6.0 (Fig. 3).

Eleven medium ingredients were selected at two levels for a Plackett-Burman experimental design (Table 2). The effects of these ingredients are

Table 4
Regression Coefficients and *t* Values of α-Amylase Production

Ingredient	Regression coefficient (b_j)	*t* Value
Intercept	3028.83	165.87
Starch	211.33	11.58
Maltose	66.42	3.64
Glucose	35.59	1.95
CSL	119.34	6.54
Soybean meal	108.25	5.93
Canola meal	13.09	0.72
NH_4NO_3	88.25	4.83
NaCl	–5.17	–0.28
KH_2PO_4	–39.67	–2.17
$MgSO_4$	–21.00	–1.15
$CaCl_2$	–11.09	–0.61

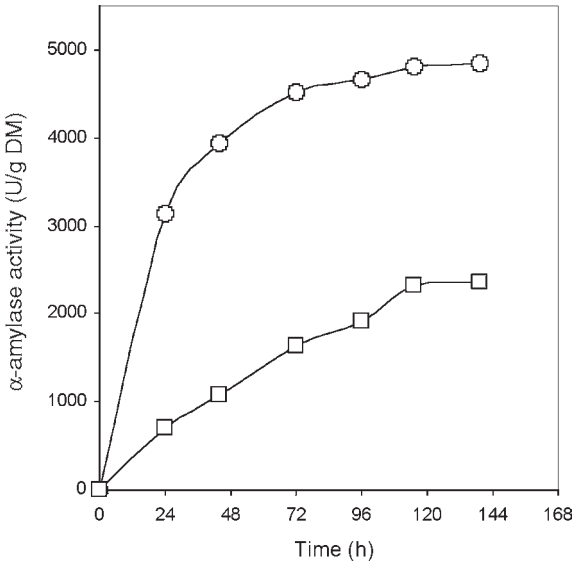

Fig. 4. Comparison of α-amylase production in nonoptimized (—□—) and optimized (—○—) solid substrate fermentation media by *A. oryzae* NRRL 1808 strain (substrate: Dreher SBG). Nonoptimized fermentation conditions: 30°C, 75% moisture content, initial pH = 6.0; optimized fermentation conditions: 25°C, 67% moisture content, initial pH = 6.0).

shown in a Pareto chart (Fig. 1). The regression coefficients and *t* values for these 11 ingredients are presented in Table 4. It can be concluded from Fig. 1 and Table 4 that starch, maltose, CSL, soybean meal, and ammonium nitrate constituted a positive effect on the production of α-amylase by the fungal culture.

The time course of fermentation was studied on both nonoptimized and optimized media (for compositions see Materials and Methods). As a

result of optimization, α-amylase activity was doubled and the fermentation time was shortened from 5 to 3 d (Fig. 4), compared with the nonoptimized fermentation.

Conclusion

SBG was transformed to a value-added product of high amylase activity by solid substrate fermentation. In a 3-d fermentation on the optimized medium, *A. oryzae* NRRL 1808 strain produced 4519 U/g DM α-amylase. The fermented product (crude enzyme, *in situ* enzyme, whole solid substrate fermentation culture) may be used advantageously without downstream processing for the hydrolysis of starchy materials and as an animal feed additive.

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References

1. Hiromi, K. (1988), *Handbook of Amylases and Related Enzymes: Their Sources, Methods, Properties and Applications*, Pergamon, Oxford, UK.
2. Bigelis, R. (1992), in *Biotechnology of Filamentous Fungi: Technology and Products*, Finkelstein, D. B. and Ball, C., eds., Butterworth-Heinemann, Boston.
3. Pandey, A., Selvakumar, P., Soccol, C. R., and Nigam, P. (1999), *Curr. Sci.* **77,** 149–162.
4. Tengerdy, R. P. (1996), *J. Sci. Ind. Res.* **55,** 313–316.
5. Pandey, A., Soccol C. R., and Mitchell, D. (2000), *Process Biochem.* **35,** 1153–1169.
6. Narahara, H., Koyama, Y., Yoshida, T., Pichangkura, S., Ueda, R., and Taguchi, H. (1982), *J. Ferment. Technol.* **60,** 311–319.
7. Tengerdy, R. P. and Szakacs, G. (2000), *Agro-Food-Ind. Hi-Tech* **11(5),** 36–38.
8. Pandey, A., Soccol, C. R., Nigam, P., and Soccol, V. T. (2000), *Biores. Technol.* **74(1),** 69–80.
9. Szakacs, G., Urbanszki, K., and Tengerdy, R. P. (2001), in *Glycosyl Hydrolases for Biomass Conversion*, Himmel, M. E., Baker, J. O., and Saddler, J. N., eds., ACS Symposium Series 769, American Chemical Society, Washington, DC, pp. 190–203.
10. May, J. B. (1987), in *Corn: Chemistry and Technology*, Watson, S. A., ed., American Association of Cereal Chemistry, St. Paul, MN.
11. Grohmann, K. and Bothast, R. J. (1997), *Process Biochem.* **32,** 405–415.
12. Sim, T. S. and Oh, J. C. S. (1990), *J. Ind. Microbiol.* **5,** 153–158.
13. Szakacs, G., Linden, J. C., Sporleder, R. A., Tengerdy, R. P., and Urbanszki, K. (1999), *Recent Advances in Fermentation Technology (RAFT III)*, Nov. 13–16, Sarasota, FL.
14. Haltrich, D., Laussamayer, B., and Steiner, W. (1994), *Appl. Microbiol. Biotechnol.* **42,** 522–530.
15. Viswanathan, P. and Surlikar, N. R. (2001), *J. Basic Microbiol.* **41(1),** 57–64.
16. Okolo, B. N., Ezeogu, L. I., and Mba, C. N. (1995), *J. Sci. Food Agric.* **69,** 109–115.
17. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
18. Stowe, R. A. and Mayer, R. P. (1966), *Ind. Eng. Chem.* **58,** 36–40.
19. Cannel, E. and Moo-Young, M. (1980), *Process Biochem.* **2,** 2–7.