ISSN 1330-9862 *original scientific paper*
(FTB-1683)

Optimization of Amylase and Protease Production from *Aspergillus awamori* in Single Bioreactor Through EVOP Factorial Design Technique

*Sangeeta Negi and Rintu Banerjee**

Agriculture and Food Engineering Department, Microbial Biotechnology and Downstream Processing Laboratory, IIT Kharagpur, West Bengal 721302, India

Received: November 16, 2005
Accepted: March 14, 2006

Summary

Evolutionary operation (EVOP) factorial design technique was explored in order to economically produce amylase and protease at their optimum level in a single bioreactor by modified solid-state fermentation. Maximum yields of amylase and protease were achieved, using wheat bran as a substrate by a highly potent, locally isolated strain of *Aspergillus awamori*: Nakazawa MTCC 6652. The strain had been induced previously, inferring the ability to produce both enzymes concomitantly in a single bioreactor with their maximum capacity. The highest secretion of amylase and protease were measured to be 9420.6 and 1930 U/g, respectively, at 37 °C. pH and relative humidity were found to be optimum at 4 and 85 %, evaluated through EVOP method.

Key words: amylase, protease, SSF, *Aspergillus awamori*, EVOP, production

Introduction

The most important industrial enzymes in use today include proteases, carbohydrate-hydrolyzing enzymes and ester cleavage fat hydrolyzing enzymes. The specific applications of such technical enzymes are in major areas of food processing, beverage production, animal nutrition, leather, paper and pulp, textiles, detergents, *etc*. Proteolytic enzymes account for nearly 60 % of the industrial enzyme market and are widely used in food industry for cheese ripening, meat tendering, the production of protein hydrolysate and bread making (*1*), and with the advent of new frontiers in biotechnology, the spectrum of amylase and protease application has expanded into many new fields, such as clinical, medicinal and analytical chemistry. To meet the current largely increased demand, studies on the cost-effective production of industrially important enzymes have become the need of today.

Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high yield of desirable enzymes. The *Aspergillus* species produces a large variety of extracellular enzymes, of which amylases and proteases are of significant industrial importance (*2*). Fungal amylases have not only been used in fermentation processes, but also in processed food industry and in the textile and paper industries (*3,4*). Considering the wide range of applicability of amylases and proteases, they are considered as the objects of our present investigation. The report of Yang and Wang (*5*) deals with the comparison of the production of protease and amylase from *Streptomyces rimosus* TM-55 in submerged (SmF) and solid-state fermentation (SSF) systems. The yields of amylase and protease were 2642.7 and 26.7 units, respectively, in solid-state cultivation. But unlike in our work, they used different substrates for the production of both enzymes. Very few other scientists have mentioned that proteases and amy-

*Corresponding author; Phone: ++91 32 22 283 104; Fax: ++91 32 22 282 244; E-mail: rb@iitkgp.ac.in, rin_tuni@yahoo.com

lases are concomitant enzymes, but never took the advantages of this fact. The yield of extracellular enzymes is significantly influenced by physicochemical conditions. Hence physical parameters must be optimized for the maximum production of enzymes through efficient optimization technique. For the first time in this paper, we have optimized the physical parameters and obtained the highest yield of amylase and protease under the same environmental conditions. For the present study, wheat bran was found to be the best substrate under SSF conditions on the basis of laboratory findings and literature survey. It is complex and has sufficient amount of carbohydrates, proteins and fibers, which is suitable for the production of both enzymes. Dimitrovski and Sapecska reported wheat bran as an appropriate substrate for α-amylase production by *Bacillus* sp. ibi-3 (*6*). On the other hand, Chakraborty and Srinivasan used wheat bran as a substrate for the production of a thermostable alkaline protease by *Pseudomonas* sp. using solid-state fermentation and got maximum activity in 96 h when the relative humidity was 74 % (*7*). SSF processes are usually simpler and can use wastes or agroindustrial substrates, such as defatted soybean cake, wheat and rice bran for enzyme production (*8*).

In the traditional biological system, optimization is done by »trial and error« or by changing one control variable at a time, while holding the rest constant. This method is inefficient in finding the true optimum conditions, especially due to the probable interaction among the factors (*9*). The EVOP methodology can be considered as a multivariable sequential search technique, in which the effect of two or three factors are studied together and the responses are analyzed statistically to arrive at the decision. The search is made sequentially, as the design of the next phase of experiments needs the results of the earlier phase of experiments (*10*).

In EVOP, decision making procedure is easily understandable and directs the change of variables towards the objective maximum or minimum value, which is not there in RSM-based factorial experiments. Apart from its simplicity, the EVOP technique has two other advantages, no deterioration in product quality and no interruption in the production of the existing plant (*11*).

Materials and Methods

Microorganism and inoculum preparation

For the present investigation, locally isolated *Aspergillus awamori*, well known for producing extracellular proteases and amylases, was used for the subsequent experiments. This strain was maintained on 2 % malt-extract agar slants at 4 °C. Wheat bran was found suitable and efficient substrate for the cost effective production of both enzymes, as it is very cheap and easily available in the market.

For inoculum preparation, 25 mL of sterile distilled water was added to the 5-day-old slant grown on malt-extract agar plate and scraped aseptically with inoculating loop. This suspension, having spore concentration of approx. $1.3\cdot10^7$ cells/mL, was used as inoculum for the subsequent fermentation.

Induced inoculum preparation

Medium for preinduced inoculum was prepared by using 1 % starch and 1 % casein in modified Czapek-Dox medium. This medium was autoclaved, then inoculated with 2 % of spore suspension containing approx. $2.5\cdot10^7$ spores from fungal strains and was incubated at 37 °C for 96 h. After the complete growth of the organism, spores were harvested by putting the flask on shaker to make a homogenous suspension.

Chemicals

The chemicals used for the experiments were all of analytical grade.

Fermentation process

The fermentation was carried out in Growtek bioreactor containing 10.0 g of wheat bran soaked in 60 mL of salt solution (Czapek-Dox (in g/L): $NaNO_3$ 2.5, KH_2PO_4 1.0, $MgSO_4\cdot7H_2O$ 0.5, KCl 0.5), which was mixed properly and autoclaved. This mixture was then inoculated with optimum number of inoculum spores aseptically. Bioreactor was then kept in a humidity chamber, where temperature and humidity were maintained during fermentation. Fermentation was conducted under various experimental conditions.

Extraction process

After complete fermentation, the fermented substrate was soaked with distilled water or other solutions for extraction of the enzymes. The extract was centrifuged at 10 000 rpm to remove the spores and other insolubles for 15 min. The supernatant was kept at 4 °C to assay the enzymes.

Amylase assay

Amylase activity was measured by Bernfeld method (1955). A reaction mixture containing 0.5 mL of 1 % (mass per volume ratio) soluble starch solution prepared in 0.2 M acetate buffer and 0.5 mL of appropriately diluted enzyme solution was incubated at 50 °C. After 10 min of incubation the reaction was terminated by adding 1.0 mL of DNS solution (1 g of DNS dissolved in 20 mL of 2 M NaOH, to which 30 g of sodium potassium tartarate were added and filled with water to 100 mL). Reaction mixtures were boiled for 15 min and after cooling, 18 mL of water were added. Absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar (glucose) in 1 min under the assay conditions (*12*).

Protease assay

Protease activity was assayed by a caseinolytic method (*13*). A volume of 2 mL of 2 % casein solution (v1) and 0.65 mL of glycine-NaOH buffer, pH=8.0 (v2) was incubated with 0.05 mL of enzyme solution (v3) at 37 °C for 20 min. After digestion, non-hydrolyzed casein was precipitated with 0.1 mL of 1 M HCl (v4) and 5 mL of 5 % TCA (v5), and removed by centrifugation at 10 000 rpm for 10 min. The protein concentration in 0.5 mL (V') of the supernatant was measured by the method of Lowry *et al.* using BSA as standard (*14*). One unit of enzyme is defined as the amount of enzyme that liberates peptide fragments equivalent to 1 mg of BSA under the assay conditions.

Calculation

The activity (Y) of enzyme protease was calculated according to the following formula: $Y=V\Delta C/V'Xv_3$ (units/mL), where $V=v1+v2+v3+v4+v5$ and ΔC=mg of peptide produced by the hydrolysis of casein by enzyme (v_3) and present in V′ (*13*).

Effect of pH, humidity and temperature on enzyme production

The pH of wheat bran was varied from 3 to 8.5 by changing the pH of Czapek-Dox. pH was adjusted using 1 M NaOH or ammonia solution and 1 M HCl before autoclaving. Humidity and temperature were controlled at different levels through humidity chamber.

Optimization of variables by EVOP based on factorial technique

The EVOP factorial technique, described earlier, was applied to select the optimum levels of three physicochemical parameters (pH, temperature and relative humidity) in different experiments. Firstly, the control experimental conditions (A1 and A6) were selected based on the results of earlier investigations on the effect of individual parameters on the production of amylase and protease by SSF. Secondly, new experimental conditions were selected with higher and lower levels of parameters compared to the control or search level. The fermentation was carried out as mentioned earlier with higher and lower levels of parameters and all experiments were repeated for two cycles. Protease and amylase yields were estimated following the procedure described earlier and recorded for cycles I and II. Differences in protease and amylase yield between cycles I and II, and average of protease and amylase yield were calculated to estimate the effects and error limits. The calculation worksheet for effects of the three variable systems is given in Table 1. The magnitude of the effects, error limits and changes in the main effect were examined as per the decision making procedure to arrive at the optimum.

Decision making procedure

After the calculation of changes in the main effects and error limits, it is necessary to examine whether any change in the control (search level) experimental condition will help to improve the objective function (*i.e.* response) and if so, which is the desired direction of change (*11*). For the above, magnitudes of the effects are compared with those of the error limits. If all or any of the effects are larger than the error limits, the change in the experimental conditions may yield better results. The decision on the desired direction of change of a variable has been described in the literature (*15*). The aim of the present investigation was to study the impact of three physicochemical factors (pH of the medium, incubation temperature and relative humidity) together in the system, on the enhancement of enzyme production by applying EVOP based on factorial technique.

Results

The most significant outcome of the present study was the maximum production of amylase and protease from *Aspergillus awamori* obtained through EVOP factorial technique using the cheapest and most easily available wheat bran as a substrate. Following the procedure described earlier, the design of three variable systems is presented in Table 1. Table 2 shows phase I of investigations, where A1 represents the »standard« or »initial optimum« conditions for three parameters, which were earlier investigated in laboratory scale to optimize them for maximum amylase and protease production. For the new sets of experiments, each variable parameter had two levels of magnitude, one higher and one lower, which were as follows: temperature (34±3) °C, pH=(5.5±2.5), and humidity (90±5) %. In Table 3, calculation of the effects and analysis of the results of cycle I for amylase production showed that the change in the main effect was large and negative (–2354.3), but all effects were not smaller than the error limits.

For amylase production, effects of temperature and pH are lower than the error limit, but the effect of humidity was higher than the error limit. Still, amylase production is maximum (9420.6 U/g) at initial optimum condition, whereas in these optimum conditions protease production was not maximum (Table 2). Therefore, the next set of experiments in phase II was performed to get protease in the maximum amount.

In phase I yield of protease was maximum at 37 °C, pH=4.0 and humidity 85 %. With this setup, the amylase yield was also considerably high; hence this setup would be the new initial optimum condition for phase II of the experimental design.

With the above-mentioned levels of parameters, the new experiments were formulated. The experimental conditions and the corresponding enzyme activities for

Table 1. Experimental design for three inducer systems

Experimental parameter	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Temperature/°C	0	–	–	+	+	0	+	–	+	–
pH	0	–	+	–	+	0	+	–	–	+
Relative humidity/%	0	–	+	+	–	0	+	+	–	–
Response	a1	a2	a3	a4	a5	a6	a7	a8	a9	a10
	→		Block I		←	→		Block II		←

0 search level, + higher level, – lower level

Table 2. Experimental conditions and results of experimental setup I for amylase and protease production

Experimental setup I	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Temperature/°C	34	31	37	37	31	34	31	37	37	31
pH	5.5	4	7	4	7	5.5	4	7	4	7
Relative humidity/%	90	85	85	95	95	90	95	95	85	85
Amylase yield (cycle I)	9256.8	7120.6	7248.0	4391.4	5214.8	9256.8	5944.8	4879.4	7585.2	7288.8
Amylase yield (cycle II)	8892.4	6476.2	8160.0	4716.8	5785.2	9420.6	5632.0	4830.6	7293.4	7653.2
Difference	364.4	644.4	–912.0	–325.4	–570.4	–163.8	312.8	48.8	291.8	–364.4
Average	9074.6	6798.4	7704.0	4554.0	5500.0	9338.7	5788.4	4855.5	7439.2	7471.0
Protease activity (cycle I)	11.66	7.26	903.2	368.3	263.6	14.15	323.9	236.8	707.8	14.72
Protease activity (cycle II)	13.98	5.46	867.0	436.7	284.68	17.08	258.0	273.9	726.7	12.0
Differences	–2.32	1.8	36.2	–68.4	–21.08	–2.93	65.8	–36.8	–18.92	2.72
Average	12.82	6.36	885.1	402.5	274.14	15.6	291.0	255.2	717.26	13.36

Table 3. Calculations of effects and limits for cycles I and II for amylase and protease

Effects	Amylase (cycle I)	Protease (cycle I)	Amylase (cycle II)	Protease (cycle II)
Effect of temperature (T)	–251.36	+418.8	6364.5	–187.97
Effect of pH (P)	+237.4	+2.65	+102.6	–4.3
Effect of humidity (R)	–1178.0	–99.78	+654.2	–77.0
Effect of TP	–955.4	+7.62	–2015.3	–52.77
Effect of TR	–688.2	–372.5	–2807.8	–55.5
Effect of PR	–231.2	–84.75	–96.2	–69.96
Change in the main effect	–2354.3	+808.14	–2700.8	–493.8
Standard deviation(s)	+335.0	+31.05	+165.3	+14.74
Error limits				
For averages	±473.8	±43.9	±223.4	±20.84
For effects	±336.4	±31.18	±165.9	±14.8
For change in the main effect	±298.5	±27.66	±2700.6	±13.13

amylase and protease are presented in Table 4. In this experiment, the change in the main effect compared to error limit is large and negative. Furthermore, most of the effects are smaller compared to the error limit and only a few effects are insignificantly larger compared to the error limit. From this analysis, it can be concluded that from the practical point of view optimum conditions have been achieved for the production of amylase and protease in single bioreactor. Finally, the yield of amylase was (7318.3+261.4) and protease was (727+26) U/g under the optimized conditions, which were higher than previously reported.

Discussion

The major objective of multiple enzyme production was aimed at cost-effective production of enzymes, which has been carried out successfully. During the optimization studies, it has been observed that the newly isolated strain is a hyperactive producer of amylases, which also produces a considerably good amount of proteases under the same experimental conditions. At pH=5.5 the amylase secretion was maximum, whereas protease was maximum at pH=4 and 7. From the observation it can be concluded that the organism is a good producer of

Table 4. Experimental conditions and results of experimental setup II for amylase and protease

Experimental setup II	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Temperature/°C	37	34	40	40	34	37	34	40	40	34
pH	4.0	3.0	5.0	3.0	5.0	4.0	3.0	5.0	3.0	5.0
Relative humidity/%	85	80	80	90	90	85	90	90	80	80
Amylase yield (cycle I)	7187.6	4452.4	1053.6	795.2	10104.5	7095.0	5906.8	411.8	775.0	8153.56
Amylase yield (cycle II)	7449.0	4351.7	1034.8	811.2	9859.4	7470.8	5796.6	470.8	729.4	8373.14
Difference	–261.4	100.68	18.8	16.0	122.55	–375.8	110.2	–59.0	165.6	219.58
Average	7318.3	4402.1	1044.2	803.2	9981.94	7282.9	5851.7	441.3	752.2	8263.3
Protease yield (cycle I)	714.0	145.0	17.5	9.05	118.76	703.6	136.48	6.02	10.57	407.0
Protease yield (cycle II)	740	156.0	10.0	7.78	96.3	725.8	144.46	4.97	14.05	379.4
Difference	–26	–11.3	+7.5	+1.27	+22.46	–22.2	–7.98	+1.05	–3.48	+27.6
Average	727.0	150.65	13.75	8.4	107.53	714.7	140.47	5.5	12.31	393.2

both enzymes in the acidic environment. Protease secretion can be boosted in the neutral condition. Temperature plays an important role in the synthesis of the enzymes. A temperature of 37 °C was found to be suitable for both amylase and protease production. At the temperature above 37 °C, as in the second experimental set-up, enzyme secretion decreased sharply, which indicated that 37 °C was the extreme for the growth of this strain. With finally achieved optimum conditions, the yield of amylase was (7318.3+261.4) and protease was (727+26) U/g, which were very high in comparision with earlier investigations.

Activity of amylase and protease was enhanced significantly by inducing the organism prior to fermentation. It was found that 1 % starch and 1 % casein solution is a good inducer medium for the production of both enzymes. Before the induction, activity of amylase and protease was 1.97-fold lower than their activity after the induction. Improvement in the enzyme yield through the induction of the strain with the help of very effortless and economical methods, without deteriorating the quality of product and organism, should be appreciated in enzyme industry. This type of efforts had not been done previously.

In EVOP method, by making a small variation around the initial optimum conditions, other conditions can be determined along with the interaction effects, which is not possible in »one at a time« optimization process (*10*). In the present study production of amylase and protease was optimized through EVOP factorial technique in which the actual optimum conditions can be predicted. Such type of optimization technique has been used for the first time to get the actual optimum data for the production of multiple enzymes in a single reactor system.

As multiple enzymes are produced from a single fermentation system, the cost of production is generally lower than the added cost of production of different enzymes produced individually from different fermentation systems. Purification of different enzymes is generally tedious and costly when they are purified individually from different fermentation systems. Stability of different enzymes in a single mixture may be doubtful when they come from different sources. With enzymes produced from a single source, less information and manipulation is required about their physicochemical behaviour. Protease and amylase are used together in many industries such as food industry, pharmaceuticals and detergent industry, *etc.* Present findings could be proved economical for enzyme industry.

Conclusions

From the results, it could be concluded that the strategy to produce amylase and protease from a single bioreactor was successful as it resulted in a considerably good amount of both of these enzymes produced by *Aspergillus awamori* in laboratory conditions. Furthermore, evolutionary operation factorial-design technique was considerably effective in maximizing the yield of both enzymes.

References

1. B. Poldermans: Proteolytic Enzymes. In: *Proteolytic Enzymes in Industry: Production and Applications*, W. Gerhartz (Ed.), VCH Publishers, Weinheim, Germany (1990) pp. 108–123.

2. A. Pandey, P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh, R. Mohan, Advances in microbial amylases, *Biotechnol. Appl. Biochem. 31* (2000) 135–152.

3. V. Ellaiah, K. Adinarayana, Y. Bhavani, P. Padmaja, B. Srinivasulu, Optimization of process parameters for glucoamylase production under solid-state fermentation by a new isolated *Aspergillus* species, *Process Biochem. 38* (2002) 615–620.

4. P. Gigras, V. Sahai, R. Gupta, Statistical media optimization and production of ITS alpha-amylase from *Aspergillus oryzae* in a bioreactor, *Curr. Microbiol. 45* (2002) 203–208.

5. S.S. Yang, J.Y. Wang, Protease and amylase production of *Streptomyces rimosus* in submerged and solid-state cultivations, *Bot. Bul. Acad. Sin. 40* (1999) 259–265.

6. A. Dimitrovski, D. Sapecska, Utilization of rice bran as substrate for alpha-amylase production by *Bacillus* sp. ibi-3, *Mikrobiologija* (Zemun), *32* (1995) 225–233.

7. R. Chakraborty, M. Srinivasan, Production of new thermostable alkaline protease by a new *Pseudomonas* sp. by solid substrate fermentation, *J. Microbiol. Biotechnol. 8* (1993) 7–16.

8. S. Germano, A. Pandey, C.A. Osaku, S.N. Rocha, C.R. Soccol, Characterization and stability of proteases from *Penicillium* sp. produced by solid-state fermentation, *Enzyme Microb. Technol. 32* (2003) 246–251.

9. A.H. Boas, What optimization is all about?, *Chem. Eng. 10* (1962) 147–152.

10. R. Banerjee, B.C. Bhattacharya, Evolutionary operation (EVOP) as a tool of optimization for solid-state fermentation, *Biochem. Eng. J. 13* (2003) 149–155.

11. R. Banerjee, B.C. Bhattacharya, Evolutionary operation (EVOP) to optimize three-dimension biological experiments, *Biotechnol. Bioeng. 41* (1993) 67–71.

12. P. Bernfeld, Amylase α/β, *Methods Enzymol. 1* (1955) 149.

13. H.E. Walter: Proteinases: Methods with Hemoglobin, Casein and Azocoll as Substrates. In: *Methods of Enzymatic Analysis, Vol. 5*, H.U. Bergmeyer, J. Bermeyer (Eds.), John Wiley & Sons Ltd., New York, USA (1984) pp. 270–274.

14. O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, Protein estimation with the Folin-phenol reagent, *J. Biol. Chem. 193* (1951) 265–275.

15. R. Tunga, R. Banerjee, B.C. Bhattacharyya, Optimization of *n*-variable biological experiments by evolutionary operation factorial-design technique, *J. Biosci. Bioeng. 87* (1999) 125–131.