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Bio-statistical approach for optimization of cold-active α-amylase production by novel psychrotolerant *M. foliorum* GA2 in solid state fermentation

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ABSTRACT

Cold-active enzymes along with their producing microbes are of commercial value and find multiple applications in various industrial and biotechnological sectors. In this study, production optimization of cold active α-amylase from cold-adapted *Microbacterium foliorum* GA2 was carried out. The aim of the present work was to use economical agro-substrate for increasing cold-active α-amylase production and to optimize the fermentation parameters in SSF (Solid state fermentation) using two statistical experimental designs. Plackett–Burman design and response surface methodology was used to determine key ingredients for the best media composition and optimal concentration of these components, respectively. The screening result of Plackett–Burman design showed bagasse, lactose and pH had significant effects (p-value ≤ 0.05) on cold-active α-amylase production. Maximum α-amylase production (6610 units) was observed through response surface methodology in medium having 40% bagasse, 0.003 M lactose, and pH 8.0 at 20 °C when incubated for 5 days in static conditions. The closeness of optimized values (R^2=92.26%) to experimental values (R^2=96.28%) proved the validity of the statistical model. Under these experimental designs, the α-amylase yield increased three-fold in comparison to control and was much efficient and economical than "one-variable-at-a-time" methodology. Thus, cold-adapted *M. foliorum* GA2 could be exploited for industrial production of α-amylase using bagasse at relatively low temperatures.

1. Introduction

Alpha-amylases are an important group of industrially and a biotechnologically relevant enzyme which is water soluble and can be found in every living organisms such as animals, plants, bacteria and fungi. But microbial sources are preferred ones due to their ability to withstand most environmental extremes such as high and low temperature and high salinity. and are relatively more stable and capable of catalyzing a variety of reactions (Burhan et al., 2003). Cold-active α-amylases of microbial origin have found immense commercial applications in various industries such as detergent industry for stain removal, in bread-baking industry as antistaling agent, in pharmaceutical industry for manufacturing of syrups, in textile industry as desizer, in paper and pulp industry for viscosity control of starch slurry, in waste water treatment and in bioremediation at low temperature. Now-a-days, commercial use of amylase is a billion dollar business that comprises a wide variety of different applications (Margesin, 2008).

To meet the demand of industries, low-cost medium is required for the production of α-amylase. Both solid state fermentation (SSF) and submerged fermentation (SmF) could be used for the production of amylases. SSF is preferred because of (a) simple technique, (b) low capital investment, (c) lower levels of catabolite repression (d) end-product inhibition, (e) low waste water output, (f) better product recovery, and (g) high quality production (Lonsane et al., 1985). For production of α-amylase in SSF, sugarcane Bagasse holds its own importance among various possible agro-substrates at industrial level (Rajagopalan and Krishnan, 2008; Roses and Guerra, 2009; Vijayabaskar et al., 2012). Sugarcane bagasse, a dietary fiber-rich by-product of the sugar industry (Sangeetha et al., 2011) corresponds to about 25% of the total weight and contains 60–80% of carbohydrates (Betancur and Pereira, 2010). The fermentation of these carbohydrates could significantly improve bioethanol productivity and sustainability but, instead, bagasse is discarded as agricultural waste or burned for energy supply in sugar and ethanol mills (Betancur and Pereira, 2010; Himmel et al., 2007; Pauly and Keegstra, 2008; Zhang and

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Table 1
Plackett–Burman experimental design matrix for screening of seven medium components for α-amylase production by *M. foliorum* GA2.

Variable level								Response
Run no.	X_1 pH	X_2 Bagasse (%)	X_3 KCl (g/L)	X_4 Yeast extract (g/L)	X_5[a]$MgSO_4$ (g/L)	X_6 Lactose (*M*)	X_7[a]Peptone (g/L)	**Enzyme activity (units)**
1	+	+	+	−	+	−	+	1300
2	−	+	+	+	−	+	−	2500
3	−	−	+	+	+	−	+	1350
4	+	−	−	+	+	+	−	1210
5	−	+	−	−	+	+	+	2245
6	+	−	+	−	−	+	+	1200
7	+	+	−	+	−	−	+	1300
8	−	−	−	−	−	−	−	1500

[pH, (-)=6.0, (+)=8.0; Bagasse (%), (-)=25, (+)=55; KCl (g/L), (-)=0.5, (+)=1; Yeast Extract (g/L), (-)=0.25, (+)=1; $MgSO_4$ (g/L), (-)=0.5, (+)=1; Lactose (M), (-)=0.002, (+)=0.004; Peptone (g/L), (-)=6, (+)=12].

[a] X5 and X7 were dummy variables.

Lynd, 2004). Both alternatives are, however, pollutant and moreover its protein deficient nature and high lignin content makes it inadequate as an animal feed (Sangeetha et al., 2011). Therefore, its utilization in one or other forms is the immediate necessity from the economic and environmental protection point of view.

In comparison to traditional method, i.e. "one-variable-at-a-time" for production of enzyme, statistically based experimental designs like Plackett–Burman design and Response Surface Methodology are more efficient in experimental biology, as variables are tested simultaneously and they need fewer experiments, which are more efficient and can move through the experimental domain. Moreover, the interactions between different variables can be estimated. The objective of this work was to select economical agro-substrates for maximum production of α-amylase by cold-adapted *M. foliorum* GA2 and to optimize the solid-state fermentation medium by using statistically based experimental designs as Plackett–Burman design and Central Composite design.

2. Materials and methods

2.1. Maintenance and growth of microorganism

A psychro-tolerant bacterium was isolated from soil of Gangotri glacier, Western Himalaya, India and was identified as *Microbacterium foliorum* GA2 in our previous study (Roohi et al., 2011). The culture was maintained on starch agar slants. The slants were incubated at 20 °C for 4 days, stored at 4 °C and sub-cultured periodically.

2.2. Screening of agro-waste by solid-state fermentation

Initial enzyme production was checked individually using agro-industrial wastes viz. wheat bran, rice husk, cassava starch, soya bean flour, sugarcane bagasse and saw-dust obtained from local area of Lucknow, India. These six agro-wastes were screened for maximum production of α-amylase and further optimization of process parameters was studied using the agro-substrate giving maximum activity with *M. foliorum* GA2 at low temperature in solid-state fermentation. Further, lactose as amylase inducer (0.002 M) was supplemented as individual component to the production media to check their effect on enzyme production (Kelly et al., 1997; Sivaramakrishnan et al., 2007).

2.3. Development of the inoculums, enzyme production and extraction

Inoculums were developed by inoculating the bacterial culture for 2 days at 20 ± 2 °C and 150 rpm and were used for further study. Cells were harvested from inoculums and their absorbance was checked at 660 nm. Accordingly, cells with inoculums size of A_{660}=0.5 [10% inoculum (v/w)] per 5 g of solid substrate (particle size, 6–8 mm) were washed, moistened with sterile distilled water in the ratio 1:1.5 (w/w) and harvested in 10 ml of basal media (gL^{-1}: peptone, 0.6; KCl, 0.05; $MgSO_4.7H_2O$, 0.05; starch, 0.1). Inoculated production media were incubated under static conditions at 20 ± 2 °C and enzyme production was checked after every 24 h. Enzyme was extracted in 50 ml of 0.1 M phosphate buffer (pH 6.0) on a rotary shaker at 250 rpm for 30 min. The content was filtered through muslin cloth, filtrate was centrifuged at 10,000 g for 10 min and clear supernatant was used as the enzyme source (Anto et al., 2006). Alpha-amylase assay was performed by the method of Swain et al. (2006). All the activity measurements were made in triplicates and experiments were repeated twice.

2.4. Enzyme assay

The amylase assay was performed as per method described by Swain et al. (2006). One unit of enzyme activity is defined as the quantity of enzyme that caused 0.01% reduction of blue color intensity of starch-iodine solution at 50 °C in 1 min per ml.

2.5. Experimental design and data analysis

2.5.1. Plackett–Burman design

The Plackett–Burman design was employed to screen the significant variables affecting cold-active α-amylase production but this design does not consider the interaction effects between the variables. The design matrix was developed according to Plackett–Burman (Plackett and Burman, 1946). The total number of experiments to be carried out according to Plackett–Burman was $N+1$, where N is the number of variables (medium components and environmental factors). Each variable was represented at two levels, namely a high level denoted by '+' and a low level denoted by '–' (Table 1). The high level of each variable was far enough from the low level so that a significant effect, if exists, is likely to be detected. Table 2 shows the Plackett–Burman design with the seven factors under investigation as well as their levels of the various factors used in the experimental design, based on the first-order polynomial model as follows:

$$Y = \beta_0 \sum \beta_i X_i \quad (1)$$

where Y is the response (growth of microorganisms), β_0 is the model intercepts, β_i is the linear coefficient and X_i is the level of the independent variable. The rows in the Table 1 represent the eight different experiments and each column represents a different variable. The conventional "one-factor-at-a-time" method was