Journal of Research and Applied Biosciences
Journal home page: www.biolresearch.com
Research Article

Protease Production using Third Generation Substrates in Solid State Fermentation by Statistical Approach: Analysis

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Article info
Received: Nov 01, 2017, Received in revised form: Nov 04, 2017, Accepted: March 10, 2018

Abstract

Solid state fermentation has numerous advantages over submerged fermentation. In solid state fermentation, various agro-industrial wastes are used as the substrate. Statistical methods are widely used to optimize various process parameters to enhance the production of biomolecules. The liquid culture medium was frequently considered as the first generation substrate for bioprocess. Later, agro-industrial wastes replaced the first generation substrate in most of the bioprocess especially the production of industrial enzymes. The third generation substrate consists of agro-wastes, including cow dung, animal feces and other effluents. The present study analyze the use of statistical optimization on enzyme production and the application of third generation substrates.

Keywords: Solid state fermentation, Response surface methodology, agro-residues, third generation substrate

Introduction

Microorganisms are widely used for the production of various biomolecules. The importance of microorganisms increased significantly to make useful products such as enzymes, nucleotides, amino acids, vitamins, organic acids, vaccines, solvents, and polysaccharides. Enzyme is an important resource utilized by the chemical, food and other industries to produce a wide range of biotechnology products and have been recognized as useful catalysts for the production of pharmaceuticals, fine chemicals and various organic transformations (Gupta et al., 2002). Among various enzymes, proteases play significant role in leather processing, neutraceutical applications, environmental applications, diagnostics and other value-added products (Rao et al. 2009).

Solid state fermentation (SSF)

Solid state fermentation (SSF) has a potential technology for the production of various biomolecules. In SSF, selections of process parameters are the important factors for optimization of enzyme production. These include nutrient factors and physiochemical parameters such as initial moisture, pH of the substrate, particle size, incubation temperature, age of the inoculum, supplementation of carbon source, nitrogen source and trace elements (Pandey, 2003). The nutritional factors such as nitrogen source, carbon source, and mineral salts are critical factors involve enzymes production. Protease comprises approximately 15% nitrogen, and its production is mainly dependent on the availability of both nitrogen and carbon sources in the culture

medium (Kole *et al.*, 1988). Although complex nitrogen sources are generally used for protease production, the requirement for a unique nitrogen supplement varies from one organism to another (Kumar and Takagi, 1999).

Several research reports have demonstrated the application of organic nitrogen sources leading to higher enzyme production than the inorganic nitrogen sources. Also, increased yields of proteases were reported by various workers, and used various sugars such as maltose, sucrose and glucose (Deepak *et al*., 2008). The divalent ions such as cobalt, calcium, copper, iron, boron, magnesium and manganese are also required in the fermentation medium for high production of alkaline proteases. Also, medium pH significantly affects various enzymatic processes and transport of many components across the cell membrane (Moon and Parulekar, 1991).

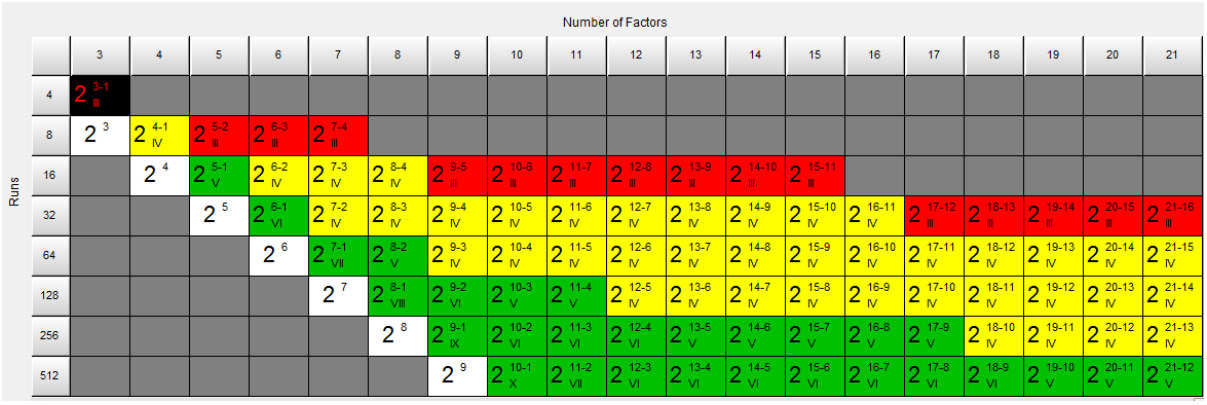
One variable-at-a-time approach – initial screening of process factors

The high production of biomolecules can be achieved by optimizing culture medium components. One variable at a time method is widely used to optimize the culture medium components. The traditional one-at-a-time optimization strategy is very simple and the effects of culture medium components can be seen on a graph individually without the need to apply any complicated statistical data analysis. However, this one-variable-at-a-time approach has some technical flaws and laborious (Vishwanatha *et al*., 2010). Statistical method of optimization is more effective and satisfactory than classical one-at-a-time approach.

Statistical optimization of enzyme production

Statistical optimization method various variables at a time with a low number of observations, saving cost and time (Myers and Montogomery, 2002). RSM is one of the such method and is highly useful for the analysis of all selected factor and their interactive effects among factors. RSM is used for the optimization and modeling of various bioprocesses, including fermentations, enzymatic reactions, product recovery and enzyme immobilization techniques (Chang *et al*., 2007). In enzyme bioprocess five or more than five factors are generally used by the researchers to optimize the process parameters. In this case, two-level fractional factorial or Plackett–Burman designs are applied to screen the process parameters. Two-level full factorial design is classified into resolutions III, IV and V. In general, full factorial designs with resolution V are widely used to screen the process parameters. In this case the required experimental run is 32. After screening with two level full factorial design, central composite design is widely applied to optimize the concentration of the process parameters. The following table shows the experimental runs and the corresponding number of factors in two level factorial experimental designs. The green colour indicates good resolution (Table 1).

Table 1. Regular two – level factorial design for the production of enzymes.



To improve and demonstrate the quality of statistical experimental model, four tests are performed: lack of fit, goodness of fit, residue analysis and statistical significance of model coefficients. Goodness-of-fit describes how well current experimental runs can be reproduced in the proposed polynomial model. Lack of fit generally allows for determining errors associated with model for experimental points are not included in basic design are similar to experimental noise or errors (Onsekizoglu *et al*., 2010). The significance of model coefficient is highly useful to identify the independent variables with real effects on response, to reduce the polynomial equation and to facilitate the inferences about the system under study (Deming and Morgan, 1993). Residue analysis is mainly performed to analyze no patterns as well as if they are distributed to assertain that residuals are randomly placed around the designed model.

In central composite rotary design (CCRD) perturbation plot is useful to find the influence of individual factors influencing bioprocess. The following figure shows the influcne of three factors (A, B and C) on enzyme production (Fig.1). Among the three factors, factor C negatively influenced on enzyme production. However, the other factors signficantly influenced on enzyme production at certain level then enzyme production decreased. In optimization experiments (e.g., CCD), the suggested experimental model should be quadratic. In poorly designed experiments, the suggested model may be linear or 2F1. In this case, it is important to redo the experiments with other center point in CCD design.

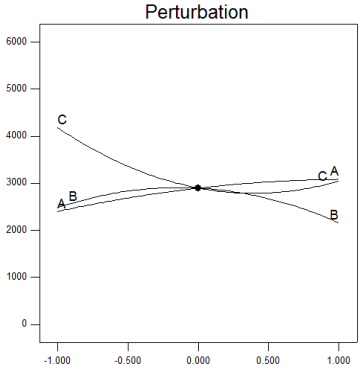


Fig. 1. Perturbation plot shows the effect of factors influencing on enzyme bioprocess.

Statistical experimental design has been commonly employed in various areas such as chemical, industrial, agricultural, engineering, food sciences and medical sciences. However, statistical experimental design is not generally used in the process optimization. The important reason for this is that most biological research has not been involved in various manufacturing processes. Since biomaterials, genetic engineering bioprocess technologies such as bioremediation and biodegradation have emerged. Biological experts very much interested in experimental designs to improve their productions and biological processes by increasing efficiencies and shortening time (Kwang-Min and David, 2005). The application of a full level factorial design and regression analysis for generating empirical models makes RSM a

good statistical tool for biomolecules production. The report on statistical approach on enzyme production in SSF is very less than submerged fermentation. This is mainly due to the technical difficulties, including, poor reproducibility of the experiments.

Third generation substrates

In recent years, efforts have been made to find the new sources to minimize the production cost of proteases using low cost feedstocks for protease production. In the first generation experiments, liquid culture medium was used for biomolecules production. Agro-industrial wastes such as, wheat bran, rice bran was considered as the second generation substrate for the production of enzymes. In the third generation, biowastes such as, cow dung, animal feces, wastewater from tannery effluent was widely studied for the production of enzymes. Cow dung was used as the substrate for the production of proteases by *Halomonas* sp. PV1 (Vijayaraghavan and Vincent, 2012), *Bacillus substilis* strain VV (Vijayaraghavan *et al*., 2012), and *Bacillus cereus* strain AT (Vijayaraghavan *et al*., 2014). Ravindran et al. (2012) applied tannery solid waste for the production of proteases from *Selenomonas ruminantium* and optimized the process parameters by RSM. Alkaline protease production by *Pseudomonas aeruginosa* was carried out using proteinaceous solid waste by Ganesh Kumar et al. (2008a). De Azeredo et al. (2006) used feather meal and corn steep liquor for the production of protease by *Streptomyces* sp. 594. Recently, animal fleshing was utilized as a potential substrate for the production of enzyme by *Clostridium limosum*. Also, enzyme production was optimized by CCRD and RSM (Ravindran et al., 2016). Goyal and Phutela (2018) used biodigested slurry for the production of proteolytic enzymes. These third generation substrates are comparatively cheap than first and second generation substrates. The search of novel substrate is a continuous process to reduce the total production cost and the third generation substrates are highly useful for the production of enzymes for various applications except food and drug use. In developed countries, the utilization of third generation substrates may help to reduce environmental pollution.

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