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ORIGINAL PAPER

Mixture design as a first step for optimization of fermentation medium for cutinase production from *Colletotrichum lindemuthianum*

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Abstract Cutinase enzymes from fungi have found diverse applications in industry. However, most of the available literature on cutinase production is related to the cultivation of genetically engineered bacteria or yeast cells. In the present study, we use mixture design experiments to evaluate the influence of six nutrient elements on production of cutinase from the fungus *Colletotrichum lindemuthianum.* The nutritional elements were starch, glucose, ammonium sulfate, yeast extract, magnesium sulfate, and potassium phosphate. In the experimental design, we imposed the constraints that exactly one factor must be omitted in each set of experiments and no factor can account for more than one third of the mixture. Thirty different sets of experiments were designed. Results obtained showed that while starch is found to have negative influence on the production of the enzyme, yeast extract and potassium phosphate have a strong positive influence. Magnesium sulfate, ammonium sulfate, and glucose have low positive influence on the enzyme production. Contour plots have also been created to obtain information concerning the interaction effects of the media components on enzyme production.

Keywords Cutinase · *Colletotrichum lindemuthianum* · Media optimization · Simplex lattice · Mixture design

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Introduction

The biotechnology industry is developing novel products in a wide range of areas such as human and animal healthcare, agriculture, environment and diagnostics. Modern tools like genetic engineering, computational analysis and computer simulation have helped to develop biological systems producing new products at a fast rate. These products vary from whole cells to extracellular secondary metabolites. However, as new systems are being developed by scientists in laboratories, engineers are faced with the task of developing the optimal media conditions for culture growth to ensure high yields and product quality. Culture medium optimization is a critical step in the fermentation process development and often continues throughout the production life of the fermentation product.

The process of developing an optimum medium for maximum production involves a stage where the critical medium components and process parameters influencing production of the desired product are screened. The primary goal in this step is to study the statistical significance of an effect that a particular factor exerts on the dependent variable of interest. Once the components critical to the production are screened, the second stage of media optimization is to find the optimum concentration of each component for maximum product formation. While developing an industrial process it is imperative to carry out the optimization studies that can be scaled up at larger scale with ease. Screening the process parameters such as nutrition components for carbon, nitrogen, phosphorus and trace elements; physico-chemical parameters such as pH, temperature, and aeration rate; both involve determining which parameters have

positive influence on production, and eliminating those having negative or no influence. Moreover, during the screening process engineers must take into consideration interactions amongst critical media components. These screening experiments can be carried out at laboratory scale with reliability, as exact concentrations are not being optimized for large-scale fermentation.

Parekh et al. [1] have reviewed the major approaches used by engineers for screening and media optimization. One of the basic approaches used to design experiments to screen media components relies on empirical and trial-and-error processes. However, statistical techniques for experimental design provide a more elegant means of designing the best medium [2]. Two of the most widely used statistical experimental designs are Plackett–Burman design, and fractional factorial design [3]. Both of these design approaches are based on factorial design of experiments, which considers the factors affecting the production process at two levels. Advantages of the designs include its simplicity and assessment of a large number of factors on the relative efficiency of the production process. However, the approaches have major drawbacks. The Plackett–Burman design does not yield estimates of the extent or type of interaction between variables [4]. In fractional design, higher-order interactions (i.e., greater than two-way) will escape detection. Also, underlying the use of two-level factors is the belief that the resultant changes in the dependent variable are basically linear in nature. This is often not the case, and many variables are related to quality characteristics in a non-linear fashion.

An alternative approach to experimental design is using mixtures. In a mixture experiment, the independent factors are proportions of different components of a blend. The interpretation of data in mixture experiments where the components represent proportionate amounts of the factors differs from classical factorial experiments where the response varies depending on the amounts of each input variable [5]. The key to mixture experiments is that the mixture components are subject to a constraint requiring that the proportions sum to one. In mixture experiments, the measured response is assumed to depend only on the relative proportions of the ingredients or components in the mixture, and not on the amount of the mixture. However, one can overcome this limitation by adding the amount of mixture as an additional factor in the experiment, thereby allowing mixture and process variables to being treated together. The advantage of mixture experiments over factorial design is that one can more efficiently study the interaction influence amongst factors on the production, and subsequently eliminate both neutral- and negative-factors. Mixture experiments have been the subject of many studies and have enjoyed extensive application in pharmaceuticals, geology, petroleum, food, and tobacco industries [6–10].

In the present study, we have used mixture design experiments to evaluate the influence of various nutrient elements on production of cutinase from the fungi *Colletotrichum lindemuthianum.* Cutinases are hydrolytic enzymes that degrade cutin, the cuticular polymer of higher plants, which is polyester composed of hydroxyl and epoxy fatty acids [11]. Cutinases have found diverse application in industrial products and processes. Hydrolytic and synthetic reactions catalyzed by cutinase have potential use in the dairy industry for the hydrolysis of milk fat, in household detergents, in the oleochemical industry, in the synthesis of ingredients for personal-care products, and the synthesis of pharmaceuticals and agrochemicals containing one or more chiral centers [12]. To date, the majority of the work has been done with a fungal pathogen of peas, *Fusarium solani f. pisi* [13–15]. Production of cutinase from this fungus is commercially unviable and thus the genes are cloned and expressed in heterologous hosts. A range of cultures have been selected as hosts to clone the cutinase gene including *E. coli* [16], *Saccharomyces cerevisiae* [17] and *Pichia pastoris* [18]. To the best of our knowledge, no information is available in the literature on the structure of cutinases produced by fungi *C. lindemuthianum* and the influence of nutrient factors on cutinase production by this or other cutinase producing fungi. Most of the production of cutinase from fungi includes culturing the organisms in potato dextrose broth. This is not an optimal medium of choice for the enzyme production. Moreover, should one envision screening various fungi cultures for cutinase having a desired property, large-scale fermentation would be needed to obtain a sufficient quantity of enzyme. The present study will help derive optimal media for future cutinase production from fungi.

Experimental

Culture and materials

C. lindemuthianum was obtained as a gift culture from Prof. P.E. Kolattukudy of the University of Central Florida, FL. All reagents and chemicals were obtained

from Sigma Aldrich Co., St Louis, MO and used as obtained.

Fermentation experiments

Fungal spores were stored in 50% glycerol at –80°C. Cell mass for fermentation experiments was developed by inoculating 100 ml of Potato Dextrose broth in a 250-ml flask with 0.1 ml of glycerol stock and incubating the medium for 8 days at 25°C under static conditions in the dark. Prior to the inoculation of the fermentation medium, the medium was vortexed to obtain a uniform cell mass.

Stock solutions of media components were prepared in de-ionized water and filter-sterilized. Using these stock solutions, fermentation media of desired compositions were prepared in 15-ml sterile tubes and the volume of the medium was adjusted to 9 ml using deionised water. One milliliter of inoculum was added to each tube and they were incubated for 12 days at 25°C under static conditions in the dark.

Enzyme analysis

At the end of incubation period, the culture tubes were centrifuged at 4,000 rpm for 30 min at 4°C. The enzyme present in the supernatant was quantified using p-nitrophenyl butyrate hydrolysis activity by a standard protocol [19]. The amount of enzyme present in the culture is reported as enzyme units, where a unit is defined as the amount of *p*-nitrophenyl formed/min/200 µl of culture supernatant. The reaction was carried out in Tris buffer at pH 8.0.

Experimental design

The primary objective of the experiment was to identify factors as either having a negative effect on enzyme production, very small or no effect, or a positive effect. Our secondary objective was to identify the three most significant factors and determine their interaction. The influence of the factors on enzyme production was studied using a simplex lattice design with constraints. The design provides an efficient method allowing us to use three levels for each factor as compared to the two levels used in popular factorial designs.

After a preliminary test case was studied with all factors at equal proportions, we imposed the constraint that exactly one factor must be omitted. In addition, no factor can account for more than one third of the mixture. Hence, the experiments consist of 30 cases, which may be partitioned into six groups each containing five cases. In each group a fixed factor is excluded from the mixture. Setting one factor at 1/3 or 33% of the mixture, and the remaining four factors at 1/6, or roughly 17% of the mixture can characterize the five cases in each group. Thus, for each factor there are three possibilities: the factor does not appear; it is 1/6 of the mixture, or 1/3 of the mixture. The experimental design generated is shown in Table 1.

Algebraically, the experimental design constraints are as follows. Let X_i denote the proportion of factor i in the mixture.

Mixture design constraint	$\sum_{i=1}^{6} X_i = 1,$
at least one factor set to 0	$X_1X_2X_3X_4X_5X_6 = 0,$
at most one factor set to 0	$X_i + X_j > 0,$
	for every pair $i \neq j,$
allowable factor levels	$X_i \in \{0, 1/6, 1/3\}.$

Analysis of data

The results were evaluated using the program Statistica (Release 6, StatSoft, USA-Tulsa). The first step was to perform a multiple regression with six independent variables representing the six factors and enzyme activity as the single dependent. The standard method was used with the intercept set equal to zero. The influence of the factors on the enzyme response is predicted by a linear polynomial of the form

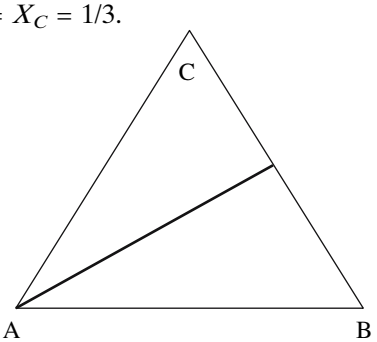
$$\text{enzyme activity} = \sum_{i=1}^{6} \beta_i X_i.$$

The factor interactions were studied using 3D-ternary plot, contour area graphs. These graphs allow one to study the interactions of any three of the factors in terms of their effect on enzyme production. The graphs given in Fig. 1 provide predicted values based on a quadratic fit. The colors are used to indicate enzyme activity. The scale for each factor follows the line segment from its corresponding vertex to the line segment opposite the vertex. For example, see the figure below where the scale for factor A has been inserted. At the vertex A, the value of X_A is assumed to be 1, and at the line segment opposite the vertex the

Table 1 Experimental design and enzyme production

Variables	Glucose	Starch	$MgSO_4$	Yeast extract	KH_2PO_4	$(NH4)_2SO_4$	Enzyme
Minimum (g/l)	0	0	0	0	0	0	
Maximum (g/l)	40	1.5	1	10	5	5	Units
Experiment							
1	0	1/3	1/6	1/6	1/6	1/6	0.76
2	0	1/6	1/3	1/6	1/6	1/6	1.30
3	0	1/6	1/6	1/3	1/6	1/6	1.68
4	0	1/6	1/6	1/6	1/3	1/6	4.77
5	0	1/6	1/6	1/6	1/6	1/3	1.43
6	1/3	0	1/6	1/6	1/6	1/6	2.60
7	1/6	0	1/3	1/6	1/6	1/6	2.04
8	1/6	0	1/6	1/3	1/6	1/6	3.93
9	1/6	0	1/6	1/6	1/3	1/6	3.23
10	1/6	0	1/6	1/6	1/6	1/3	2.75
11	1/3	1/6	0	1/6	1/6	1/6	1.26
12	1/6	1/3	0	1/6	1/6	1/6	1.85
13	1/6	1/6	0	1/3	1/6	1/6	4.75
14	1/6	1/6	0	1/6	1/3	1/6	2.50
15	1/6	1/6	0	1/6	1/6	1/3	1.58
16	1/3	1/6	1/6	0	1/6	1/6	1.37
17	1/6	1/3	1/6	0	1/6	1/6	2.00
18	1/6	1/6	1/3	0	1/6	1/6	0.67
19	1/6	1/6	1/6	0	1/3	1/6	9.07
20	1/6	1/6	1/6	0	1/6	1/3	1.18
21	1/3	1/6	1/6	1/6	0	1/6	1.55
22	1/6	1/3	1/6	1/6	0	1/6	2.06
23	1/6	1/6	1/3	1/6	0	1/6	2.54
24	1/6	1/6	1/6	1/3	0	1/6	2.00
25	1/6	1/6	1/6	1/6	0	1/3	2.10
26	1/3	1/6	1/6	1/6	1/6	0	2.88
27	1/6	1/3	1/6	1/6	1/6	0	0.78
28	1/6	1/6	1/3	1/6	1/6	0	1.93
29	1/6	1/6	1/6	1/3	1/6	0	3.00
30	1/6	1/6	1/6	1/6	1/3	0	4.33

factor is 0. The values for the other two factors along this scale for A satisfy $X_B = X_C$. The point where all three factor scales intersect has co-ordinates $X_A = X_B = X_C = 1/3$.



Mixture surface regression was also used to study the interactions of groups of three factors and obtain prediction equations for enzyme with an associated R-value. The prediction equation is a quadratic of the form

$$\text{enzyme activity} = \sum_{i=1}^{3} \beta_i X_i + \sum_{i<j} \sum^{3} \beta_{ij} X_i X_j.$$

Among all ${}_6C_3 = 20$ groups of three factors, the group that provides the best prediction for enzyme production in terms of largest R-value is glucose (denoted by X_g), yeast extract (X_y) and KH_2PO_4 (X_k). The prediction equation is given below.

$$\begin{aligned}\text{Enzyme} = {} & 0.1348\ X_g + 0.0173\ X_y + 0.4538\ X_k \\ & + 1.3110\ X_g X_y - 1.4970\ X_g X_k \\ & + 0.3930\ X_y X_k.\end{aligned}$$

Results and discussion

To understand the interactions of nutrients in the medium on cutinase production by *C. lindemuthianum*, six medium components were selected viz. glucose, starch (Carbon sources), ammonium sulfate (Nitrogen source), yeast extract (source for Nitrogen and trace elements), potassium phosphate (Phosphate source) and magnesium sulfate (Magnesium source). These ingredients and the maximum values for each of them

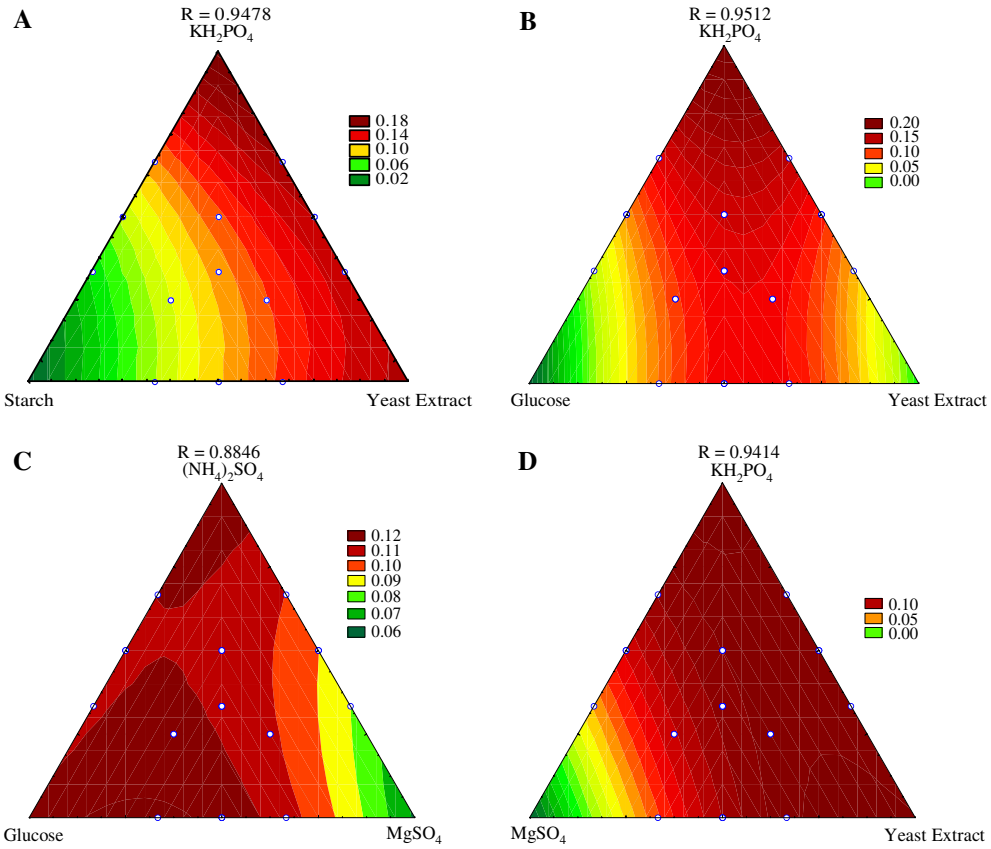


Fig. 1 Quadratic contour area ternary plots of various media ingredients. (Units are expressed as increase in absorbance observed in the study in two minutes by 200 µl of culture medium)

were selected after screening the literature on various types of media used to cultivate fungi for enzyme production. In mixture experiments, it is necessary to have the sum of all ingredients equal to one (100%). However, one cannot design experiments where the concentration of each media components equals one. To overcome this, we express the concentration of each medium component in a given experiment in terms of a fraction of the maximum value.

As shown in Table 1, the mixture experimental design requires conducting 30 experiments to carry out the screening. A factorial design would require $2^6 = 64$ experiments to screen the six media components. Alternatively, a fractional factorial design requiring $2^{6-1} = 32$ cases could be employed. In general, if we have n factors to test for screening, the mixture design employed here requires $n(n-1)$ experiments. Hence, the growth in the number of experiments is quadratic as opposed to exponential growth in the experiments required of a factorial design.

Table 2 Results of the analysis of results using multiple regression

Multiple regression coefficient $R = 0.9451$		
Factor	β_i	P level
Glucose	0.1625	0.1596
Starch	–0.1032	0.3659
$MgSO_4$	0.0394	0.7277
Yeast extract	0.4843	0.0002
KH_2PO_4	0.4330	0.0007
$(NH_4)_2SO_4$	0.0223	0.8436

Results obtained on cutinase production were analyzed using multiple regression analysis. The general purpose of multiple regression is to learn more about the relationship between several independent or predictor variables and a dependent variable. Upon analysis, it was found that starch has a negative influence on enzyme production (Table 2). Starch has beta value of –0.1032 indicating that an increase in starch concentration decreases the enzyme production. Of the other ingredients, yeast extract and potassium phosphate showed a strong positive influence on cutinase production. Yeast extract had the lowest P value (0.0002) and the highest positive beta value (0.4843), indicating that a change in the concentration of yeast extract will increase enzyme production. Previously it has been reported that some organic and inorganic compounds present in the yeast extract can induce extracellular enzyme production in fungi, probably by

inducing the intracellular production of cAMP [20]. A similar influence could be hypothesized to be exerted by yeast extract on cutinase production by *C. lindemuthianum.* Potassium phosphate also has a strong influence on cutinase production (beta 0.433; $P = 0.0007$). This indicates that phosphate is the limiting nutrient parameter in cutinase production. Ammonium sulfate has the lowest positive beta value and highest P value indicating that cutinase production in this fungus is not dependent on the concentration of the nitrogen source. Similarly, magnesium sulfate also had a minimal impact on enzyme production. In the concentration range tested in this experimental design, glucose was found to have weak influence on enzyme production.

To obtain information concerning the interaction of media components on enzyme production, contour plots were created. Figure 1a–d illustrates how different media components interact with each other to influence enzyme production. Figure 1a, supports the conclusion that increasing starch concentration decreases the enzyme production. In addition, maximum enzyme production is predicted when high concentrations of yeast extract and potassium phosphate are present. Interesting interaction results are observed in Fig. 1b. When three positive parameters obtained using multiple regression are plotted against each other, it is projected that when glucose and yeast extract concentration are increased from zero, enzyme production increases until we reach the centre of triangle. However, upon further increase in concentration of either parameter, a sharp decrease in enzyme production is predicted. It should be noted that at the vertices where a maximum concentration of glucose or yeast extract is present, there is complete absence of potassium phosphate. This strongly supports our earlier observation that phosphate is a key factor in enzyme production. It can also be observed that the absence of potassium phosphate must be accompanied by absence of either glucose or yeast extract for complete absence of cutinase production. Ammonium sulfate has almost no influence on enzyme production (Fig. 1c). Also, near the vertices where maximum magnesium sulfate is present, decreased enzyme production is predicted (Fig. 1c, d). This could be because of either an inhibition of cutinase production in the presence of excess concentration of magnesium sulfate, or complete absence of glucose or yeast extract and interactions with other factors.

Based on the above results we conclude that yeast extract, potassium phosphate and glucose are factors that are statistically significant and should be included in further experimentation for medium optimization for cutinase production by *C. lindemuthianum.* Also, through the present study we have demonstrated that mixture experiments are ideal to screen medium ingredients and to understand the interactions amongst them.

References

1. Parekh S, Vinci VA, Strobel RJ (2000) Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol 54:287–301
2. Strobel RJ, Sullivan GR (1999) Experimental design for improvement of fermentations. In: Demain AL, Davies JE (eds) Manual of industrial microbiology and biotechnology. Am Soc Microbiol. Washington, pp 80–93
3. Broedel SE Jr, Papciak SM, Jones WR (2001) The selection of optimum media formulations for improved expression of recombinant proteins in *E. coli.* Technial Bull 2:1–6
4. Motola S, Agharkar SN (1992) Preformularion research of parental medications. In: Avis KE, Liberman HA, Lachman M (eds) Pharmaceutical dosage forms: parental medications. Marcell Decker, New York, pp 115–172
5. Chu JS, Amidon GL, Weiner WD, Goldberg A. (1991) Mixture experimental design in the development of a mucoadhesive gel formulation. Pharmaceutical Res 8:1401–1407
6. Cornel JA (1981) Experiments with mixtures: designs, models and the analysis of mixture data. Wiley, New York
7. Aitchison J (1986) The statistical analysis of compositional data. Chapman and Hall, New York
8. Anik ST, Sukumar L (1981) Extreme vertexes design in formulation development: Solubility of butoconazole nitrate in a multicomponent system. J Pharm Sci 70:897–900
9. Johnson AD, Anderson VL, Peck GE (1990) A statistical approach for the development of an oral controlled-release matrix tablet. Pharm Res 10:1092–1097
10. Cornell JA, Gorman JW (1984) Fractional design plans for process variables in mixture experiments. J Qual Technol 16:20–38
11. Purdy RE, Kolattukudy (1975) Hydrolysis of plant cutin by plant pathogens. Purification, amino acids composition, and molecular weight of two isoenzymes of cutinase and nonspecific esterase from *Fusarium solani f. pisi.* Biochemistry 14:2824–2831
12. Carvalho CML, Aires-Barros MR, Cabral JMS (1998) Cutinase structure, function and biocatalytic applications. Electronic J Biotechnol 1
13. Lin TS, Kolattukudy PE (1980) Structural studies on cutinase, a glycoprotein containing novel amino acids and glucuronic acid amide at the N terminus. Eur J Biochem 106:341–351
14. Dantzig AH, Zuckerman SH, Andonov-Roland MM (1986) Isolation of a *Fusarium* mutant reduced in cutinase activity and virulence. J Bacteriol 168:911–916
15. Murphy CA, Cameron JA, Huang SJ, Vinopal RT (1996) *Fusarium* polycaprolactone depolymerase is cutinase. Appl Environ Microbiol 62:456–460
16. Lauwereys M, De Geus P, De Meutter J, Stanssens P, Mathyssens G (1991) Cloning, expression and characterization of cutinase, a fungal lipolytic enzyme. In: Alberghina L, Schmid RD, Verger R (eds) Lipases-structure, function and genetic engineering. VCH Weinheim, USA, pp 243–251

17. Sagt CMJ, Verrips CT (1995) Secretion of wild-type and mutant cutinases by *Saccharoyces cerevisiae*. Yeast 11: Special Issue, p 594
18. Gross R (2006) Expression of *Fusarium solanii* cutinase gene in *Pichia pastoris* (Personal communication)
19. Shirai K, Jackson RL (1982) Lipoprotein lipase-catalyzed hydrolysis of p-nitrophenyl butyrate. Interfacial activation by phospholipid vesicles. J Biol Chem 257:1253–1258
20. Baracat-Pereira MC, Coelho JLC, Minussi RC, Chaves-Alves VM, Brandão RL, Silva DO (1999) Cyclic AMP low molecular weight effector (s) present in yeast extract are involved in pectin lyase production by *Penicillium griseoroseum* cultured in sucrose. Appl Biochem Biotechnol 76:129–141