Experimental design of medium optimization for invertase production by *Pichia* sp.

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Abstract The culture medium requirement for invertase production by *Pichia* sp. was optimized and identified by initial screening method of Plackett–Burman. Furthermore, optimum concentrations of medium components, which were selected by in initial screening by Plackett–Burman, were determined by the Box–Behnken and its representative three-factor response-surface method. The regression models showed significantly high R^2 values of 97% for invertase activities, indicating that they are appropriate for predicting relationships between yeast extract, peptone and sucrose concentration with invertase production. According to the model the optimal concentrations of sucrose, yeast extract and peptone were 40, 5 and 4 g/ml, respectively. These predicted conditions were verified by validation experiments. In the optimized medium *Pichia* sp. produced invertase with activity of 38.71 U/ml, which is 4 times higher than that produced in original medium. Thus, this statistical approach enabled rapid identification and integration of key medium parameters for *Pichia* sp. BCCS M1, resulted the high invertase production.

Keywords Box–Behnken · Experimental design · Optimization of invertase · Plackett–Burman · *Pichia*

Introduction

Invertases (EC.3.2.1.26) or β -fructofuranosidases are special kind of enzymes that catalyze the hydrolysis of sucrose into a mixture of glucose and fructose named inverted sugar (Ahmed 2008; Chavez et al. 1997). Sucrose hydrolysis may carried out either by hydrochloric acid at 75–80 °C or by invertase enzyme at 35–45 °C with a notorious advantage for an enzymatic over acid processes in terms of energy economy, environmental safety and low formation of by-products (Gines et al. 2000). The enzyme has wide range of commercial and biotechnological applications in beverage, the production of confectionery with liquid or soft centre, bakery and other pharmaceutical formulations for the preparation of inverted sugar and high fructose syrup (HFS) from sucrose. It also contributes fermentation of cane molasses into ethanol (Gehlawat 2001). Moreover, microbial invertase is used for the manufacture of calf feed and food for honeybees (Vandamme and Derycke 1983).

Most investigations related to invertase production have been performed by means of one-factor-at-a-time technique (Kuar and Sharma 2005; Shafiq et al. 2003a, b). This optimization methodology is not always powerful strategy for obtaining optimal medium because of potential interactions among medium components. Moreover, following the conventional strategy, large numbers of experiments are required for identifying the optimal levels of all medium components (Chuan-He et al. 2007). Optimizing all the effecting parameters can eliminate these limitations of a single factor optimization process collectively by statistical experimental design using Plackett–Burman and Box–Behnken design methodology. Plackett–Burman design is a well-established and widely used statistical design method for the screening of the nutritional components of medium in shake flask (Mee 2009; Vanot and Sergent 2005). The

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design screens the important variables effecting the invertase production as well as their significance levels but does not consider the interaction effects among the variables. The variables screened by Plackett–Burman design were further optimized in a three-level factorial Box–Behnken design methodology (Kalil et al. 2000; Strobel and Sullivan 1990).

To date no studies on medium engineering for invertase production of *Pichia* have been reported. Thus, this report tries to formulate an appropriate production medium using statistical optimization that can substantially enhance the invertase production by *pichia*.

Materials and methods

Isolation and screening of microorganisms

The microorganism used in this study, was isolated from a soil sample collected from field area (sugar beet farmland, Shiraz, Iran) and selected based on the ability to grow in the selective medium containing sucrose as a sole source of carbon and energy source at 30 °C. The isolation media had the following composition (per liter of distilled water): (NH₄)₂SO₄, 0.5 g; MgSO₄*7H₂O, 0.2 g; KH₂PO₄, 3.0 g; mineral salts solution, 2 ml; and sucrose (E. Merck AG), 20 g at pH 7.0. The medium containing of (g/l) sucrose 30.0; peptone 5.0 and yeast extract 3.0 at pH 6.5 was used for screening of isolated invertase producing microorganism.

Identification of microorganisms

The morphological, physiological, and biochemical characterizations of the invertase-producing isolate were determined by identification tests. For further identification, the analysis of 18S rRNA gene sequence was performed according Ghasemi et al. (2008). Briefly: an aliquot (1 mL) of cultured cells (in mid to late exponential phase) was harvested by centrifuge (13000g for 3 min at room temperature) in a sterile 1.5 mL microcentrifuge tube. Pelleted cells were resuspended in 0.5 mL of PBS buffer. The mixture was shaken and the genomic DNA was extracted by heat shock method and used for PCR amplification of 18S rRNA gene. Amplified DNA samples were electrophoresed in a 1% (w/v) agarose gel using TBE electrophoresis buffer containing 1 µg/mL ethidium bromide. The gel was photographed under UV light. The molecular weight of the PCR amplified product was calculated using Major Sciences gel documentation system. The two oligonucleotide primers used for amplification of yeast 18S rRNA gene were: The universal eukaryotic primers 5'-GTCAGAGGTGAAATTCCTGGATTTA-3' as forward primer and 5'-AGGGCAGGGACGTAATCAACG-

3' as reverse primer (prepared by Aran Sanat Yekta, Tehran, Iran), which amplify a ~700-bp region of the 18S rRNA gene. PCR reaction was performed in a total volume of 50 µL containing 10 µL of chromosomal DNA in TE buffer pH 8.0 and 2 µL of each primer. PCR products were purified and then sequenced by CinnaGen Company (Tehran, Iran). The resulting 18S rRNA gene sequences were aligned and compared to the nucleotide sequences of some known microorganisms in GenBank database of the National Center for Biotechnology Information by using Basic Local Alignment Search Tool (BLAST). The nucleotide sequences of 18S rRNA genes were deposited to GenBank under the accession numbers HQ735406.

Seed culture preparation

Cell suspension was prepared from 2 to 3-days-old slant cultures. Twenty-five ml of seed medium was transferred to each 250 ml Erlenmeyer flask. The medium consisted of (g/l) sucrose 30.0; peptone 5.0 and yeast extract 3.0 at pH 6.5, unless stated otherwise. The flasks were cotton plugged and autoclaved at 103.5 Pa pressure (121 °C) for 15 min and cooled at room temperature. One ml of inoculum was transferred to each flask under sterile conditions. Flasks were then incubated overnight in a rotary incubator shaker at 30 °C. Agitation rate was kept at 140 rpm (Shafiq et al. 2003b).

Fermentation technique

Production of invertase was carried out by the shake flask technique using 250 ml Erlenmeyer flasks containing 100 ml of medium. A 10% (v/v) microorganism suspension was transferred from an overnight seed culture to the production medium composed of (g l⁻¹): peptone, 5; yeast extract, 3; (NH₄)₂SO₄, 0.05; sucrose, 30 and pH 6.5 (Shafiq Ali and Ul-Haq 2003). For the purpose of the experimental design, the production medium was prepared in different formulae as illustrated in Table 1. Flasks were then incubated in a rotary incubator shaker at 30 °C for 48 h. The agitation rate was kept at 140 rpm. After 48 h, the cells were removed by centrifugation and the supernatants were used for measurement of invertase activity.

Invertase activity assay

Enzyme activity assay was determined according to the method of Miller (1959) with a few modifications as follows. The enzyme activity assay was carried out in a solution containing 150 µl of enzyme solution which was mixed with 150 µl of 0.3 M sucrose on 0.05 M acetate buffer, pH 4.5. The mixture was maintained at 37 °C for 1 h, and the rate of appearance of inverted sugar was

Table 1 Plackett–Burman design and invertase activity after 48 h of fermentation

Trial num	(NH ₄) ₂ SO ₄ (g/l) X_1	pH X_2	Yeast extract (g/l) X_3	Peptone (g/l) X_4	Sucrose concentration (g/l) X_5	Activity (U/ml)
1	0.5 (−1)	6 (−1)	5 (+1)	7 (+1)	40 (+1)	35.0222
2	1 (+1)	6 (−1)	2 (−1)	4 (−1)	40 (+1)	19.3361
3	0.5 (−1)	6 (−1)	2 (−1)	4 (−1)	20 (−1)	11.7963
4	1 (+1)	7 (+1)	2 (−1)	7 (+1)	20 (−1)	14.4425
5	1 (+1)	6 (−1)	5 (+1)	4 (−1)	20 (−1)	30.9745
6	0.5 (−1)	7 (+1)	5 (+1)	7 (+1)	20 (−1)	31.6033
7	1 (+1)	7 (+1)	2 (−1)	7 (+1)	40 (+1)	22.0404
8	0.5 (−1)	7 (+1)	2 (−1)	4 (−1)	20 (−1)	15.0451
9	0.5 (−1)	6 (−1)	2 (−1)	7 (+1)	40 (+1)	20.6824
10	1 (+1)	6 (−1)	5 (+1)	7 (+1)	20 (−1)	33.3063
11	1 (+1)	7 (+1)	5 (+1)	4 (−1)	40 (+1)	31.3195
12	0.5 (−1)	7 (+1)	5 (+1)	4 (−1)	40 (+1)	30.1667

The values in parenthesis are the levels of coded variables

determined by the dinitrosalicylic acid method. One unit of invertase activity is defined as the amount of enzyme that hydrolyzes 1 μ mol of sucrose/min under the aforementioned conditions.

Optimization procedure

The optimization of medium constituents for invertase production by *Pichia* sp. was carried out in two stages. All experimental designs and data analysis were done by MINITAB software (version 15, PA, USA).

Identification of important nutrient components

Plackett–Burman design (Plackett and Burman, 1946), which examines high and low levels of different experimental variables, has been used to screen and identify important medium components for product yield was followed as shown in Table 1. The source and concentration of carbon and nitrogen were varied to determine which combination provided the highest invertase yield. A total of 12 variables were tested: sucrose as a carbon source was tested as 20 and 40 g l^{-1} , while nitrogen sources (yeast extract, peptone and (NH₄)₂SO₄) were tested as 2 and 5 g l^{-1} , 4 and 7 g l^{-1} , 0.5 and 1 g l^{-1} respectively. Moreover, the pH value was tested as 6 and 7. Each component was added in different combinations according to Table 1.

Plackett–Burman experimental design is based on the first order model:

$$Y = \beta_0 + \sum \beta_i X_i$$

where Y is the response (enzyme activity), β_0 is the model intercept and β_i is the linear coefficient, and X_i is the level of the independent variable. This model does not describe

interaction among factors and it is used to screen and evaluate the important factors that influence the response (Table 2). In the present work, five assigned variables were screened in twelve experimental designs. All experiments were carried out three times and the averages of the invertase activity were taken as response.

Optimization of screened components

The next stage in medium optimization was to determine the optimum level of each key independent variable as identified by the Plackett–Burman design. An experimental design such as the Box–Behnken design (Box and Behnken, 1960), which is a fraction of the full factorial, was used. Unlike the Plackett–Burman design, this design evaluated the quadratic effects and two-way interactions among the variables and thus determined the nonlinear nature of the response, if any. The design for the three independent variables is shown in the Table 3. For predicting the optimal point, a second order polynomial function was fitted to correlate relationship between independent variables and response (invertase activity). For the three factors this equation is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_1 \beta_2 X_1 X_2 + \beta_1 \beta_3 X_1 X_3 + \beta_2 \beta_3 X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Where Y is the predicted response, β_0 model constant; X_1 , X_2 and X_3 independent variables; β_1 , β_2 and β_3 are linear coefficients; β_{12} , β_{13} and β_{23} are cross product coefficients and β_{11} , β_{22} and β_{33} are the quadratic coefficients. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2 . Experiments were performed in triplicate and mean

Table 2 Statistical analysis of Plackett–Burman design showing coefficient values, *t*- and *P*-values for each variable on invertase activity

Variables	Effect	Coefficients	<i>t</i> - value	<i>P</i> -value
Constant		1.606	0.20	0.850
(NH ₄) ₂ SO ₄	4.735	2.368	1.04	0.337
pH	−2.167	−1.083	−0.96	0.376
Yeast extract	9.894	4.947	13.09	0.000 *
Peptone	2.051	1.025	2.71	0.035 *
Sucrose concentration	0.357	0.178	3.15	0.020 *

* are significant values at *p*<0.05

values are given. Finally, the final optimum experimental parameters were calculated using the Minitab Response Surface Optimizer function, which allows for identifying the best combination of each component.

Results and discussion

Isolation and screening of invertase producing microorganisms

The microorganism plate screening on solid selective medium containing sucrose as a sole carbon and energy source yielded 10 colonies after 2 days of incubation. These 10 microorganisms were screened for invertase activity when the strains were grown on basal broth medium. Experiments were repeated thrice and the average values were applied. The results showed that the activity of strain BCCS M1 were all higher than those of other nine strains (data not shown), suggesting that strain BCCS M1 was a valuable strain with higher invertase activity.

Identification of the isolate strain

To identify the isolated strain, the morphological, physiological, and biochemical tests were carried out and the results showed that all these properties of this invertase-producing microorganism were similar to those of yeast *Pichia*. Finally, according to the widely used method of 18S rRNA gene analysis, we cloned and sequenced the 18S rRNA gene of this invertase-producing isolate strain, named as BCCS M1 (18S rRNA accession no. HQ735406). The almost complete sequence of 18S rRNA gene showed 95% homology with *Pichia* strain (GenBank:, HM151325, HM151318 and so on). Therefore, according to observations and analyses, strain BCCS M1 was classified as *Pichia*.

Screening of important media components for invertase production by *Pichia* sp. BCCS M1

Invertase activities following the Plackett–Burman design were measured at 24 and 48 h of fermentation; however,

Table 3 Experimental and predicted values of invertase recorded in Box-Behnken design

Trial	Yeast extract (g/l) <i>X</i> ₁	Peptone (g/l) <i>X</i> ₂	Sucrose concentration (g/l) <i>X</i> ₃	Experimental activity (U/ml)	Predicted activity (U/ml)
1	3.5 (0)	5.5 (0)	30 (0)	28.8948	27.2897
2	2 (−1)	5.5 (0)	40 (+1)	14.7438	13.6610
3	3.5 (0)	7 (+1)	40 (+1)	27.2357	26.7225
4	3.5 (0)	5.5 (0)	30 (0)	28.3768	27.2897
5	3.5 (0)	5.5 (0)	30 (0)	24.5974	27.2897
6	5 (+1)	7 (+1)	30 (0)	32.9054	32.5020
7	5 (+1)	5.5 (0)	40 (+1)	35.1942	36.1108
8	3.5 (0)	4 (−1)	40 (+1)	29.2648	29.9442
9	3.5 (0)	4 (−1)	20 (−1)	12.9594	13.3628
10	5 (+1)	5.5 (0)	20 (−1)	22.2534	23.3362
11	5 (+1)	4 (−1)	30 (0)	33.7524	32.1564
12	2 (−1)	4 (−1)	30 (0)	13.9594	13.9628
13	2 (−1)	5.5 (0)	20 (−1)	12.9553	12.0387
14	2 (−1)	7 (+1)	30 (0)	15.9524	17.5484
15	3.5 (0)	7 (+1)	20 (−1)	25.6906	25.0112

The values in parenthesis are the levels of coded variables

only data at 48 h were considered because at this time all trials showed higher activities (data not shown). Table 1 represents the Plackett–Burman experimental design for 12 trials with two levels of concentrations for each variable and corresponding invertase activity. Activities varied according to the fermentation conditions, from 11.80 to 35.02 U/mL. The comparisons of invertase production in different media are given in the Table 1, shows that the medium in trial 1 gave the highest enzyme yield, followed by medium in trial 10 and 6. The best result was achieved with the following conditions: sucrose concentration of 40 gL⁻¹, yeast extract concentration of 5 gL⁻¹, peptone concentration of 7 gL⁻¹, (NH₄)₂SO₄ concentration of 0.5 gL⁻¹ at pH 6.0. According to these results, this variation reflects the importance of medium optimization to obtain higher efficiency. The main effects of the examined factors on the enzyme activity were calculated and presented in Fig. 1. According to analysis of the regression coefficients of the five variables, all variables had positive effect on invertase activity except pH that contributed negatively (Table 2). The polynomial model describing the correlation between the five factors and the invertase activity could be presented as follows:

$$Y_{\text{activity}} = 1.606 + 2.368X_1 - 1.083X_2 + 4.947X_3 \\ + 1.025X_4 + 0.178X_5$$

according to calculated *t*-values and *P*-values (Table 2), yeast extract, peptone and sucrose found to be the most significant variables influencing invertase activity. The insignificant variable, pH has negative effect on invertase production; though it will be kept at 6 value in order to bringing maximum invertase activity. Depending on the invertase producing species, various optimal pH values ranging from 6 to 8, were reported in literature from

invertase (Chavez et al. 1997; Gines et al. 2000; Gogoi et al. 1998). Application of an appropriate nitrogen source was very important for optimal production of invertase, because sucrose metabolism shows a specific physiological response to the presence of nitrogen source (Hocine et al. 2000; Shafiq et al. 2003a, b). Among organic and inorganic nitrogen sources, it was revealed that organic nitrogen source (yeast extract and peptone), has the most influence effect on invertase activity than (NH₄)₂SO₄ as an inorganic nitrogen source. This insignificance effect of (NH₄)₂SO₄ may due to libration of ammonium ions, that could declined the enzyme activity as reported by other researchers (Kuar and Sharma 2005; Vandamme and Derycke 1983).

Other variables with less significant effect were not applied to next optimization experiment, but instead were used in all trials at their (−1) level and (+1) level, for the negatively contributing variables and the positively contributing variables, respectively, which keeping at inferior levels had a lower cost of production in comparison to the superior level.

Optimization of screened medium components for invertase production by *Pichia*

The variables that showing *P*-value below 0.05 in the Plackett–Burman design were opted and further optimized using Box–Behnken design. Contour plots and surface plots were obtained when the data (of invertase production) were incorporated to the MINITAB software, and analyzed by it. The software has the function, which we can predict the production of invertase within studied range of all three medium components. Here each contour plot and surface plot represents the effect of two medium components at their studied concentration range and at fixed concentration of the third medium component

Fig. 1 Pareto chart rationalizing the effect of each variable on the invertase activity (U/ml) produced by *Pichia* sp.BCCS M1

Fig. 2 Response surface plot and contour plot of the combined effects of yeast extract (X_1) and sucrose concentration (X_3) on the invertase production by *Pichia* sp. BCCS M1

(Figs. 2, 3 and 4). Table 4 represents the design matrix of the coded variables together with the experimental results of the invertase activity. All cultures were performed in triplicate and the average of the observations was used. Data were analyzed by linear multiple regression using the MINITAB software and the following equation was obtained.

$$Y = 27.2897 + 8.4368X_1 + 1.1328X_2 + 3.5992X_3 - 3.4223X_1^2 + 0.0251X_2^2 - 2.5806X_3^2 - 0.9600X_1X_2 + 2.7881X_1X_3 - 2.7436X_2X_3$$

Where, Y is the predicted response and X_1 , X_2 and X_3 are the coded values of yeast extract, peptone and sucrose, respectively. As listed in Table 4, the high F -value and the very low P -value implied that the experimental model was in good agreement with the experimental. The ANOVA represented that the linear term of the polynomial model was much significant. Value of lack of fit F and lack of fit p -

value were found to be 0.66 and 0.649, respectively, which implies that the lack of fit is non-significant. Non-significant lack of fit made the model fit. The coefficient of determination ($R^2=0.97$) in the experimental model suggested an excellent agreement between experimental results and their predictions. The R^2 value is always between 0 and 1. The closer the R^2 value to 1 suggest the stronger model, therefore it is better to predict the responses (Alam et al. 2010).

The response surface/contour plots of calculated model for invertase production are shown in Figs. 2, 3 and 4. All the response surfaces/contour could be analyzed for determining the optimized value of the factors, but it was difficult to analyze all these simultaneously. Hence, the final optimal levels of the three components were estimated using the Minitab Response Surface Optimizer function and found to be: yeast extract 5; peptone 4 and sucrose 40 g l^{-1} . This model also predicted activity of 38.71 U/ml for invertase. The excellent correlation between predicted and observed values of the Box–Behnken (Table 4) justifies the

Fig. 3 Response surface plot and contour plot of the combined effects of yeast extract (X_1) and peptone (X_3) on the invertase production by *Pichia* sp. BCCS M1

validity of the response model and the existence of an optimum point.

Most authors had studied the production of invertase by bacteria and a few studies in yeast (Ahmed 2008; Gines et al. 2000; Hara et al. 1990; Ikram-ul-Haq and Sikander 2005; Shafiq et al. 2003a, b; Vullo et al. 1991). Ikram-ul-Haq et al. studied invertase production by *Saccharomyces cerevisiae* using one-factor-at-a-time technique. These authors studied various nitrogen sources and sucrose concentrations on invertase production. Based on Ikram-ul-Haq and Sikander (2005) studies, it was revealed that 3 g l⁻¹ of yeast extract and 30 g l⁻¹ of sucrose concentration gave high invertase production. Shafiq et al. found 4 g l⁻¹ of peptone concentration for optimum invertase production which is similar to our achievements. However, no data was encountered concerning optimization of invertase production by *Pichia*, using experimental design. Instead, there are several studies of another kind of β -fructofuranosidase enzyme; inulinase, by *Pichia*, which hydrolysis inulin (Gong et al. 2007). These authors investigated various organic and inorganic nitrogen and carbon sources on inulinase production. Their studies were showed that yeast

extract and peptone as an organic nitrogen source had profound effect on inulinase production than ammonium sulfate and ammonium chloride as an inorganic nitrogen source.

Verification of model

The optimized values of nutrient parameters (yeast extract 5; peptone 4 and sucrose 40 g l⁻¹) were validated in a triplicate shake flask study and an average 36.6 U/ml of invertase production was obtained. The verification was satisfactorily close to the predicted of the model for more than 94%, which is an evidence for the model validation under the investigated circumstances.

Conclusion

The conditions for invertase production by *Pichia* sp. BCCS M1 were optimized by two-stage of the statistical experimental designs, named Plackett-Burman and Box-Behnken. These experimental methodologies revealed an

Fig. 4 Response surface plot and contour plot of the combined effects of peptone (X_2) and sucrose concentration (X_3) on the invertase production by *Pichia* sp. BCCS M1

increase in invertase production of new isolated yeast strain BCCS M1, by medium engineering. As an economic point of view, the most important factors in screening and optimization of media components are cost and time. The strategy used here demonstrates advantages in comparison with traditional methods and allows the development of a mathematical model that predicts

where the optimum is likely to be located. After 2 experimental designs and 27 experiments, optimized conditions for invertase production by *Pichia* sp. BCCS M1 were obtained. The best fermentation condition was shown to be: 40 g/l of sucrose, 5 g/l of yeast extract, 4 g/l peptone, which resulted in an enzymatic activity of 38.71 U/ml.

Table 4 ANOVA for the model that represents invertase activity from *Pichia*

Source	DF	sum of squares	mean of squares	F-value	P-value
Regression	9	812.19	90.243	20.60	0.002
Linear	3	683.34	227.780	52.00	0.000
Square	3	63.96	21.321	4.874	0.061
Interaction	3	64.89	21.630	4.94	0.059
Residual Error	5	21.90	4.380		
Lack-of-Fit	3	10.89	3.632	0.66	0.649
Pure-Error	2	11.01	5.503		
Total	14	834.09			

DF degrees of freedom;
 $R^2=0.97$

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