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Use of a sequential strategy of experimental design to optimize the inulinase production in a batch bioreactor

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Abstract A potential application of inulinase in the food industry is the production of fructoligosaccharides (FOS) by the transfructosilation of sucrose. The FOSs present many interesting functional properties besides their ability to increase the shelf-life and flavor of many products. The use of an industrial medium represents a good alternative to producing inulinase at low cost, since the activity may improve, or at least remain the same, as that obtained using a synthetic medium. This work was an optimization study of the inulinase production by *Kluyveromyces marxianus* NRRL Y-7571 using industrial pre-treated culture medium in a bioreactor employing a sequential strategy of experimental design. Initially, a Plackett–Burman (Screening Design) design was used, where the studied variables were molasses, corn steep liquor, yeast extract concentration, and agitation and aeration rates. After the analysis of the effects, a central composite rotational design (CCRD) was carried out. The optimized condition for the inulinase production was: 250 g/l of molasses, 80 g/l of corn steep liquor, 6 g/l of yeast extract, 300 rpm of agitation and 1.5 vvm aeration rate, which resulted in an enzymatic activity of 1,317 ± 65 U/ml.

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Introduction

Fructose and fructooligosaccharides are fast emerging as important ingredients in the food industry. Fructose is considered to be a safe alternative sweetener to sucrose because it shows beneficial effects in diabetic patients, increases iron absorption in children and has a higher sweetening capacity. Fructooligosaccharides have functional and nutritional properties for use in low-calorie diets, for stimulation of *Bifidus* and as a source of dietary fiber in food preparations [1]. Both fructose and fructooligosaccharides can be produced by inulinase via the enzymatic hydrolysis of inulin, the latter being a fructose polymer found in plants, such as Jerusalem artichoke, chicory and dahlia [2, 3].

Inulinase can be extracted from many plants, but the yield is low, increasing productivity costs [4–6]. The possibility of using microorganisms to produce enzymes represents a good alternative to increasing productivity, and in the present case, this would increase the potential for using inulinase in the industrial production of fructose from inulin [7]. Several papers have reported the production of inulinase by biotechnological processes using synthetic and industrial media [8, 9].

In recent years, research on the selection of suitable substrates for fermentative processes has mainly been centered on agro-industrial residues because of their potential advantages. In addition, the utilization of these agro-industrial wastes, on the one hand, provides alternative substrates and, on the other, helps in solving pollution problems, which otherwise may cause their disposal. The nature of the substrate employed is the most important

factor affecting fermentative processes, and its selection depends upon several factors mainly related with cost and availability and, thus, may involve the screening of several agro-industrial residues [10].

The utilization of agro-industrial residues as substrate in bioprocesses is a time-consuming task and requires going through some stages. Firstly, it is necessary to select a microorganism that grows in these complex mediums and excretes the product of interest. For inulinase production, the yeast *Kluyveromyces marxianus* NRRL Y-7571 has shown good adaptation and productivity in mediums containing molasses and corn steep liquor, as previously reported by our research group [11]. Secondly, the downstream process should be viable technically and economically, considering the limiting step. Some authors demonstrated that the pre-treatment of the substrates before their use in fermentation, in addition to improving production, also has possibilities for the direct purification of the fermentation broth for the inulinase separation [12]. Thirdly, higher productivities are only obtained after process optimization.

The use of the sequential strategy of the experimental design is a useful tool for process optimization, particularly when it involves a considerable number of variables [13]. Xiong et al. [14] employed a sequential strategy of experimental design to optimize the inulinase production in solid-state fermentation. Initially, a PB with 12 runs was carried out to evaluate the influence of 8 independent variables. After the analysis of the effects, only three independent variables were investigated in a Box–Benken design, where the process optimization was carried out by the realization of only 27 runs.

In the present work, the optimization of inulinase production by *Kluyveromyces marxianus* NRRL Y-7571 in a batch bioreactor, using industrial pretreated culture medium, was carried out using a sequential strategy of the experimental design, considering the following variables: molasses, corn steep liquor, yeast extract concentration, agitation rate and aeration rate. These five parameters were previously evaluated in a Plackett–Burman design (12 runs plus 3 central points). Subsequently, a central composite rotatable design (CCRD) was performed (11 runs) to establish the optimal conditions for inulinase production.

Materials and methods

Pretreatment of molasses and corn steep liquor

Molasses was obtained from a local sugar refinery (Éster Sugar Refinery Company, Campinas SP, Brazil). Corn steep liquor (CSL) was obtained from Corn Products Brazil (Mogi Guaçu Sp, Brazil). The method used to pre-treat molasses and CSL in order to reduce harmful compounds, which can act as growth inhibitors during the fermentation process, was as follows: 1 h of incubation time, 70°C of incubation temperature and 8% w/v active carbon type ANF (Carvorite Factory, Irati PR, Brazil), maintained under agitation. Following the activated carbon treatment, the substrate was then centrifuged at 10,000 rpm for 15 min at 5–10°C.

Fermentation

Flask culture

Kluyveromyces marxianus NRRL Y-7571 was employed for inulinase production since it meets the requirements of GRAS ("Generally Recognized as Safe") and is accepted by the FDA (Food and Drug Administration). The microorganism was grown on MY broth. The inoculum cultures were grown on a medium containing 2% sucrose with pH at 6.5 in 500-ml flasks with 100 ml of culture medium at 30°C and 150 rpm for 48 h.

Bioreactor

The fermentations were carried out in a Bioflo III (New-Brunswick Scientific) fermenter containing 2.2 l of the pretreated culture medium. Corn steep liquor and molasses had been pretreated using active carbon separately. The fermentations were started with 10% (V/V) inoculum at initial pH 5.0 (no pH control) and 36°C for 72 h according to a previous work by our research group [15]. A pitched-up blade impeller was used for agitation.

Sequential strategy of experimental design

The choice of the best strategy for the experimental design is a function of the number of independent variables or factors in question and of the initial knowledge about the process. Normally, when the optimization process is started, the operational conditions are distant from the global optimum. Then it is preferable to realize a screening design, such as Plackett–Burmam (PB), to adjust the levels or select the significant variables and afterwards to develop a new screening design or central composite rotational design (CCRD). Therefore, the effects analysis as initial information can be sequentially targeted by the new screening design until a CCRD is reached in which the desired conditions are obtained [13].

Plackett–Burman design

Plackett–Bruman design (PB) is a very useful tool for picking the most important factors from a list of candidate

factors. At this early problem-solving stage, the methodology assumes that important main effects will be much larger than two-factor interactions, so we were willing to confound main effects with two-factor interactions [16]. Therefore, this technique was used to identify the more important independent variables, to verify if the investigated levels were in adequate range and to select them to realize another fractional design or a complete factorial design. The influence of five variables on enzymatic activities was investigated using this methodology. The effects of molasses, corn steep liquor (CSL), yeast extract concentration, agitation and aeration rates were studied. Table 1 shows the values of coded levels used.

Central composite rotational design (CCRD)

It was shown by the analysis of the effects in the Plackett–Burman design that only two variables, molasses and corn steep liquor (CSL) concentration, were significant. As the variables yeast extract concentration, aeration rate and agitation rate were not significant in the investigated range, for the next experimental design they were maintained at 6 g/l, 1.5 vvm and 300 rpm, respectively. In order to describe the nature of the response surface in the optimum region, a CCRD for two independent variables was performed, totaling 11 experiments [16, 17]. The independent variables were molasses concentration (180–320 g/l) and CLS concentration (50–110 g/l). The software Statistica 6.0 (Statsoft Inc., USA) was used to analyze the results.

Inulinase assay

Activity was assayed according Silva-Santisteban and Maugeri [8] as follows: 1 ml enzyme solution was mixed with 9 ml of 2% (w/s) sucrose or inulin on acetate buffer 0.1 M pH 4.5. The mixture was maintained at 50°C, and the rate of appearance of fructose was determined by the DNS method [18]. One unit of inulinase activity is defined as the amount of enzyme necessary to hydrolyze 1 μmol of sucrose per min under the conditions mentioned above (sucrose as substrate).

Table 1 Values of coded levels and real values used in the Plackett–Burman design

Coded variable levels	−1	0	+1
Corn steep liquor (g/l)	50	60	70
Molasses (g/l)	150	180	210
Yeast extract (g/l)	6	7	8
Agitation rate (rpm)	300	400	500
Aeration rate (vvm)	1.5	2.0	2.5

Microbial growth

The microbial growth was determined by an indirect dry weight method, by comparing the cell suspensions optical density at 600 nm to a standard curve, which was previously prepared with the same microorganism produced in the same culture medium.

Results and discussion

Plackett–Burman design

The first experimental design was performed in order to screen the relevant variables in inulinase production. Table 2 shows the results of the Plackett–Burman design. As can be seen, the inulinase activity ranged from 300 to 1,198 U/ml, according to the fermentation conditions at 72 h. The best result was achieved with the following conditions: molasses concentration of 210 g/l, corn steep liquor concentration of 70 g/l, yeast extract concentration of 8 g/l, agitation rate of 300 rpm and aeration rate of 2.5 vvm in run 6.

According Haaland [19], the P values are useful for screening experiments, but it is probably better to accept higher P values, say $P < 0.10$, rather than to take the chance of missing an important factor. In this work, the variables that had effects on inulinase activity were molasses ($P < 0.0001$) and corn steep liquor concentration ($P = 0.0263$). The positive effects showed that an increment in both levels of concentrations led to an increase in

Table 2 Plackett–Burman design and inulinase activity after 72 h of fermentation

Trial	CSL	Molasses	Yeast extract	Agitation	Aeration	Activity (U/ml)
1	1	−1	1	−1	−1	399
2	1	1	−1	1	−1	1,001
3	−1	1	1	−1	1	830
4	1	−1	1	1	−1	400
5	1	1	−1	1	1	1,110
6	1	1	1	−1	1	1,198
7	−1	1	1	1	−1	998
8	−1	−1	1	1	1	310
9	−1	−1	−1	1	1	305
10	1	−1	−1	−1	1	345
11	−1	1	−1	−1	−1	997
12	−1	−1	−1	−1	−1	300
13	0	0	0	0	0	601
14	0	0	0	0	0	640
15	0	0	0	0	0	629

the inulinase activity. The yeast extract concentration ($P = 0.7810$), aeration rate ($P = 0.9913$) and agitation rate ($P = 0.8424$) were not significant, indicating that range studied of these variables does not affect the inulinase activity. Then, in the next experimental design, these variables were fixed at the inferior level (6 g/l, 300 rpm and 1.5 vvm for yeast extract concentration, agitation rate and aeration rate, respectively) and had a lower cost of production compared with the superior level.

CCRD

Molasses and corn steep liquor concentrations were selected and studied using a CCRD and response surface analysis. The runs were carried out to obtain a second order model in order to predict the inulinase activity as functions of corn steep liquor and molasses concentration. According to Table 3, the best results for the inulinase activity were in trials 9, 10 and 11, all of them corresponding to the central points, with molasses concentration of 250 g/l and corn steep liquor concentration of 80 g/l. The inulinase activity was 1,317 ± 65 U/ml (mean of three triplicates). To compare the results with the medium without pre-treatment, three experiments were carried out in triplicate in conditions with the central point of the CCRD obtaining a mean activity of 980 ± 25 U/ml. Probably the pre-treatment reduced the concentration of some inhibitor compounds present in the medium and increased the production. Valduga et al. presented the complete composition of CSL and molasses before and after treatment with active carbon. After the pre-treatment a considerable reduction occurred in the concentration of minerals, such as zinc, manganese, copper, iron, magnesium, potassium and phosphorus, among others. These minerals, when present at high concentration, could inhibit microbial growth [20].

Other studies concerning inulinase production by *Kluyveromyces* in submerged fermentations showed that the amount of enzyme produced varied greatly with the strain and the kind of nutrient supplementation used. Some authors obtained maximum production of 7.0 U/ml using fructanes as the carbon source for *Kluyveromyces fragilis* [7]. Kalil et al. [21] used sucrose as the carbon source and obtained 127 U/ml of inulinase by *Kluyveromyces marxianus*. Silva-Santisteban and Maugeri [8], using factorial design and response surface analyses, optimized the inulinase production with sucrose as carbon source and obtained the maximum of 176 U/ml. The response surface method was used by Wei et al. [9] to optimize the medium for inulinase production, and the maximum inulinase produced was 68.9 U/ml with extracts of *Jerusalem artichoke*, urea, beef extract and corn steep liquor at concentrations of 8.0, 2.0, 0.2 and 4.0%, respectively.

For testing the goodness of fit of the regression equation to the date of the inulinase production, the values at 72 h of fermentation were considered. The analysis of variance (ANOVA) of a quadratic model presented a determination coefficient, R^2, of 0.9150, indicating that 91.5% variability in response could be explained by the model. The calculated F was six times higher than the listed F (4.35), at a 95% confidence level. Therefore, this model can predict the inulinase activity, in U/ml, using molasses concentration (M) and corn steep liquor concentration (CSL). The coded model was good enough to describe the response surface of inulinase activity (Eq. 1).

$$\text{Activity} = 1316.7 - 394.5 \times \text{M}^2 - 131.4 \times \text{CSL} - 292.6 \times \text{CSL}^2 \quad (1)$$

The model represented by Eq. 1 for inulinase activity was used to generate the response surfaces, which can be seen in Fig. 1. Since all coefficients of the above equation are negative, the response surface is suggested to have a maximum point. The corn steep liquor and

Table 3 Matrix of the results obtained in the CCRD for the inulinase activity at 72 h of fermentation

Trial	Molasses (g/l)	CSL (g/l)	Experimental activity (U/ml)	Predicted activity (U/ml)
1	200 (−1)	69 (−1)	612	761
2	300 (1)	69 (−1)	830	761
3	200 (−1)	101 (+1)	512	498
4	300 (1)	101 (+1)	712	498
5	180 (−1.41)	80 (0)	480	532
6	320 (+1.41)	80 (0)	501	532
7	250 (0)	50 (−1.41)	989	920
8	250 (0)	110 (+1.41)	400	550
9	250 (0)	80 (0)	1,380	1,317
10	250 (0)	80 (0)	1,320	1,317
11	250 (0)	80 (0)	1,250	1,317

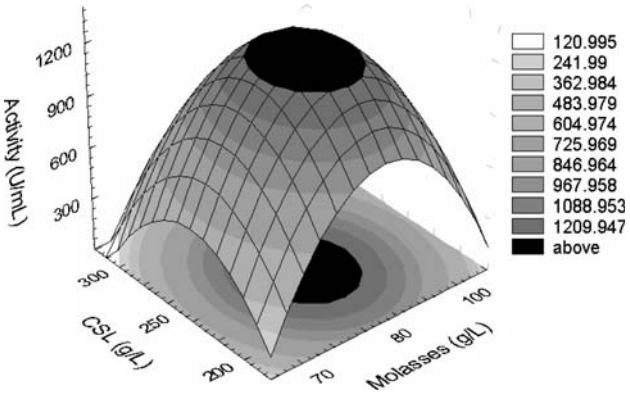

Fig. 1 Response surface for the inulinase activity as functions of molasses and corn steep liquor concentration

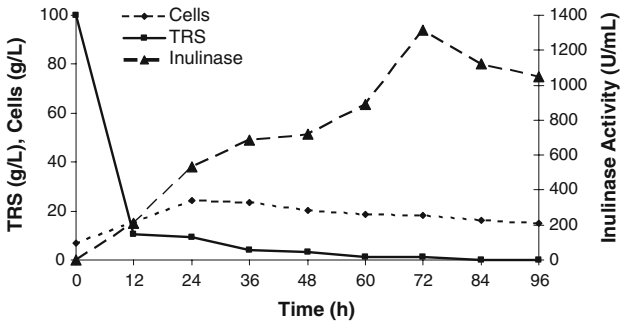

Fig. 2 Kinetics of inulinase production at optimum condition

molasses showed significant negative effects. This result supports the idea that agro-industrial residues have complex compositions where a substrate can show a negative effect in an overall system due to an increment in the concentration of some constituent. In the case of corn steep liquor, the nitrogen source probably increased, resulting in inhibition because of excess substrate.

The optimal concentrations for the molasses and CSL were calculated to be 250 and 80 g/l, respectively. The model predicted the response of 1,317 U/ml of inulinase at this point. The excellent correlation between predicted and measured values at the central point of the CCRD (Table 3) justifies the validity of the response model and the existence of an optimum point.

One of the most important aspects of using agro-industrial residues for the production of bio-products is related to the optimal concentration of these substrates. Since considerable variation may occur in the composition of these complex substrates, a region for the maximum production is desirable and not a single specific concentration. An analysis of the response surfaces and contour curves showed a range of concentration for the maximum enzyme concentration, which is important since it allows for greater variability in the raw material without decreasing production.

After optimization, fermentation kinetics was realized at the optimized conditions as observed in Fig. 2. The experimental errors are lower than 5% for the all experimental points in the Fig. 2. The results showed that in the first 12 h of fermentation a drastic decrease in the total sugar concentrations occurred, with the total sugar decreasing to about zero after 72 h of fermentation. The inulinase production started at about 10 h of fermentation, achieving a maximum point at 72 h of fermentation and decreasing after this period.

Inulinase productivity obtained in our work was 18.1 U/(ml h), almost seven times the highest productivity reported in the literature for submerged fermentation, 2.4 U/(ml h) [8], and almost three times higher productivity for solid state fermentation, 6.2 U/(g h) [22].

Conclusions

The sequential strategy of the experimental design was demonstrated to be very useful for the optimization of inulinase production, mainly because of the large number of factors considered. After 2 experimental designs and 26 experiments, optimized conditions for inulinase production by *Kluyveromyces marxianus* NRRL Y-7571 using industrial pretreated culture medium were obtained. The best fermentation condition was show to be: 250 g/l of molasses, 80 g/l of corn steep liquor, 6 g/l yeast extract, 300 rpm of agitation and 1.5 vvm of aeration, which resulted in an enzymatic activity of 1,317 ± 65 U/ml. The highest productivity obtained in this work showed that it is possible to use industrial medium (molasses and corn steep liquor) at low cost, instead of inulin or other kinds of fructan. This is a very interesting alternative since inulin is only available in limited quantities and at high cost.

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