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Inulinase Production by *Kluyveromyces marxianus* NRRL Y-7571 Using Solid State Fermentation

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Abstract

Inulinase is an enzyme relevant to fructose production by enzymatic hydrolysis of inulin. This enzyme is also applied in the production of fructo-oligosaccharides that may be used as a new food functional ingredient. Commercial inulinase is currently obtained using inulin as substrate, which is a relatively expensive raw material. In Brazil, the production of this enzyme using residues of sugarcane and corn industry (sugarcane bagasse, molasses, and corn steep liquor) is economically attractive, owing to the high amount and low cost of such residues. In this context, the aim of this work was the assessment of inulinase production by solid state fermentation using by *Kluyveromyces marxianus* NRRL Y-7571. The solid medium consisted of sugar cane bagasse supplemented with molasses and corn steep liquor. The production of inulinase was carried out using experimental design technique. The effect of temperature, moisture, and supplements content were investigated. The enzymatic activity reached a maximum of 445 units of inulinase per gram of dry substrate.

Index Entries: Inulinase; solid state; *Kluyveromyces marxianus*.

Introduction

Inulinases (E.C. 3.2.1.7) are glycosidases that perform the endohydrolysis of 2,1-β-D-fructosidic linkages in inulin. These enzymes are potentially useful in the production of high fructose syrups, using inulin as substrate (*1*). The production of fructose from inulin is more advantageous than the conventional method that uses starch, given that the reaction using inulinase is simpler and yields products with fructose content higher than 95%. Inulinases have also been used for production of fructo-oligosaccharides (*2*). Researches involving fructo-oligosaccharides have been stimulated by

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the increase in the demand for healthy foods (3), because the ingestion of fructoligosaccharides increases the production of *Bifidobacterium*, which are the most important bacteria of intestinal microflora (4).

Several microorganisms may be used to produce inulinase. The selection of such microorganisms depends on their physiological characteristics and the fulfillment of Generally Recognized as Safe (GRAS) and Food and Drug Administration (FDA) criteria for food products (5). The gender *Kluyveromyces* satisfies these requirements, which makes this microorganism interesting for commercial applications. *Kluyveromyces marxianus* NRRL Y-7571 has shown excellent results in submerged medium in the experience we have gathered in collaboration with the Laboratory of Bioprocesses in UNICAMP (results not shown). Several studies about inulinase production can be found in literature. Most of them focus on submerged fermentation systems and other microorganisms (6–10). However, few reports about inulinase production using solid state fermentation (SSF) can be found (11). The use of low cost residues, higher productivities, low energy requirements, lower wastewater production, extended stability of products, and low production costs are some of the main advantages of SSF (12,13). The use of SSF presents the possibility of using low cost substrate, like agroindustry wastes and bioproducts, then adding value to such materials (14). This work examined the production of inulinase by *K. marxianus* by SSF, using sugar cane bagasse as substrate and sugar cane molasses (SCM) and corn steep liquor (CSL) as of carbon and nitrogen supplementary sources.

Material and Methods

Microorganism

K. marxianus NRRL Y-7571 obtained from NRRL (Northern Regional Research Laboratory, now the National Center For Agricultural Utilization Research, Peoria, IL was maintained in Agar slants at 5°C on YM Agar medium (Yeast Malt) containing (g/L) 3, 3, 5, 10, and 20 g/L of yeast extract, malt extract, peptone, glucose, and agar, respectively.

Preinoculum preparation was carried out inoculating 10 mL of liquid YM medium in a 50-mL tube with a loopful of stock culture, and incubating at 30°C, for 24 h. Inoculum medium contained: 20 g/L sucrose, 5 g/L yeast extract, 5 g/L K_2HPO_4, 1.5 g/L NH_4Cl, 1.15 g/L KCl, and 0.65 g/L $MgSO_4 \cdot 7H_2O$ at initial pH 6.8. All reagents and medium components were analytical grade and supplied by Vetec Ltd. (Rio de Janeiro, RJ, Brazil). Each tube with liquid YM medium was transferred to a 500 mL Erlenmeyer containing 100 mL of medium and then incubated at 30°C and 150 rpm for 24 h.

Solid State Fermentation

Sugar cane bagasse from a local industry was used as substrate for inulinase production. Fermentations were carried out in conical reactors using 5 g of dry bagasse, supplemented with CSL and SCM in the concentrations

Table 1
Coded Levels and Real Values Used in the First Complete Factorial Design (2^4)

Coded variable levels	–1	0	+1
Temperature (°C)	30	35	40
Moisture (%)	50	65	80
Molasses (%)	0	5	10
Corn steep liquor (%)	0	5	10

Table 2
Coded Levels and Real Values Used in the Second Complete Factorial Design (2^3)

Coded variable levels	–1.68	–1	0	+1	+1.68
Temperature (°C)	31.6	35	40	45	48.4
Moisture (%)	39.8	50	65	80	90.2
Corn steep liquor (%)	6.6	10	15	20	23.4

defined in the experimental design. All flasks were sterilized at 121°C for 20 min. Each reactor was inoculated with 3 mL of the inoculum cell suspension obtained previously and incubated for 72 h. Cultivation was performed in an incubator with humidified air injection. All experimental conditions were carried out in duplicate. Control reactors were also carried out to discount enzyme activity already present in the inoculum. In control runs the enzyme activity was determined right after inoculation. The effect of temperature of incubation, initial moisture of the substrate, and supplement concentrations on inulinase production was assessed by a complete 2^4 experimental design. Table 1 presents the range of the factors investigated. Details on experimental design technique are described by Rodrigues and Iemma *(15)*. After analysis of the results of the first experimental design using a Experimental Design Analysis software tool (Statistica® 5.1 Statsoft Inc, Tulsa, OK), a second experiment was performed based on a central composite design with two factors and six axial points. The range of the factors that were investigated is presented in Table 2.

Enzyme Extraction

After incubation, enzyme was extracted adding 50 mL of sodium acetate buffer 0.1 *M* pH 4.5 to the fermented medium, following incubation at 30°C for 30 min at 150 rpm and filtration with Whatman qualitative paper, no. 3. Enzyme activity was assayed in the supernatant after filtration.

Inulinase Assay

Inulinase activity was assayed as follows: 1 mL enzyme solution, obtained after extraction, was mixed with 9 mL of 2% (w/v) sucrose on sodium acetate buffer 0.1 *M* and pH 4.5. The mixture was maintained at

Table 3
Matrix of the First Experimental Design With Coded Levels and Real Values (in Parenthesis) for the Inulinase Activity

Run	Moisture (%)	Molasses (%)	CSL (%)	Temperature (°C)	Activity (U/g)
1	–1 (50)	–1 (0)	–1 (0)	–1 (30)	n.d.
2	+1 (80)	–1 (0)	–1 (0)	–1 (30)	45
3	–1 (50)	+1 (10)	–1 (0)	–1 (30)	9
4	+1 (80)	+1 (10)	–1 (0)	–1 (30)	n.d.
5	–1 (50)	–1 (0)	+1 (10)	–1 (30)	n.d.
6	+1 (80)	–1 (0)	+1 (10)	–1 (30)	29.6
7	–1 (50)	+1 (10)	+1 (10)	–1 (30)	n.d.
8	+1 (80)	+1 (10)	+1 (10)	–1 (30)	60.1
9	–1 (50)	–1 (0)	–1 (0)	+1 (40)	n.d.
10	+1 (80)	–1 (0)	–1 (0)	+1 (40)	n.d.
11	–1 (50)	+1 (10)	–1 (0)	+1 (40)	n.d.
12	+1 (80)	+1 (10)	–1 (0)	+1 (40)	n.d.
13	–1 (50)	–1 (0)	+1 (10)	+1 (40)	n.d.
14	+1 (80)	–1 (0)	+1 (10)	+1 (40)	113.8
15	–1 (50)	+1 (10)	+1 (10)	+1 (40)	11.1
16	+1 (80)	+1 (10)	+1 (10)	+1 (40)	78.6
17	0 (65)	0 (5)	0 (5)	0 (35)	n.d.
18	0 (65)	0 (5)	0 (5)	0 (35)	n.d.
19	0 (65)	0 (5)	0 (5)	0 (35)	0.5

n.d., not detected.

50°C and the rate of appearance of fructose was determined by the dinitrosalicylic acid (DNS) method *(16)*. One unit of inulinase activity is defined as the amount of enzyme that hydrolyses 1 μmol of sucrose per min, under the reaction conditions.

Results and Discussion

Table 3 presents the matrix of the first complete experimental design with central points and inulinase activity as response. The highest inulinase activity (114 U/g) was obtained using the substrate with 80% of moisture added of 0% of molasses, and 10% of CSL, and incubated at 40°C (run 14). We observed that the extracts present very good stability at room temperature (results not shown).

The data presented in Table 2 were statistically treated and the effects of the factors on inulinase activity are presented as the Pareto chart shown in Fig. 1. Moisture and CSL concentration were the factors that most influenced inulinase activity, with a positive significant effect ($p < 0.05$). The positive effect of moisture is probably related to higher water requirements of yeasts when compared with fungi, which are most commonly used in SSF. CSL is a complex supplement, which is a good source of nitrogen

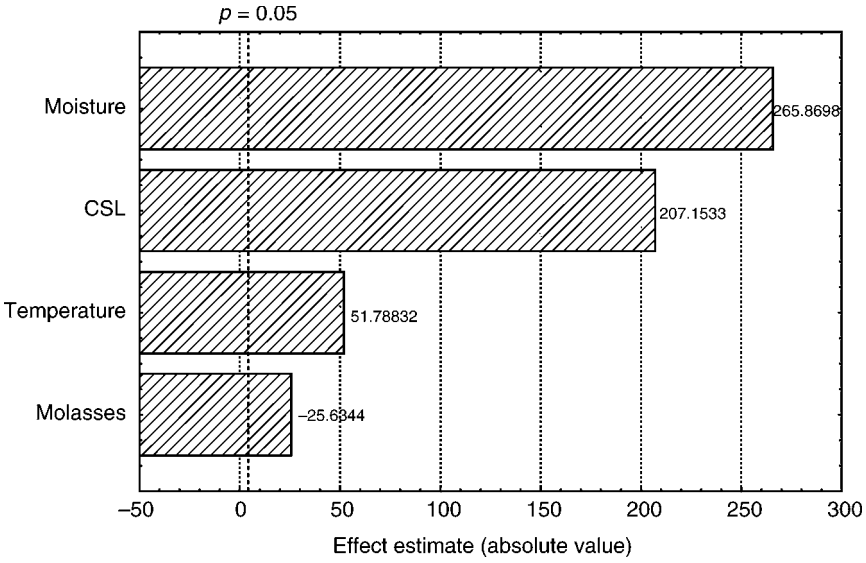

Fig. 1. Pareto chart of effects for the first experimental design.

and micronutrients. The positive effect of the concentration of this supplement on inulinase activity shows that the amount of CSL added in the substrate should be improved to satisfy the microorganism nutrient requirements.

The results obtained in the first experimental design show that the use of molasses as supplement is unnecessary, because this factor presented a negative significant effect. Other possibility is the presence of heavy metals in the molasses, which would inhibit enzyme production by the microorganism *(6)*. Therefore, a second experimental design was carried out, aiming to optimize inulinase production. The ranges of the factors studied were adjusted based on the results of the first experimental design. Table 4 presents the results of the second complete experimental design. Maximum inulinase activity obtained in this work was 445 U/g, obtained at the axial point (40°C, 65% moisture, 23.4% CSL). An increase in inulinase production can be noticed when results of second experimental design are compared with the results of the first one. Coded models for inulinase activity as a function of the studied factors could not be validated for any of group of experimental data.

Statistical analysis of the second experimental design yielded the Pareto chart presented in Fig. 2. It is worth noting that CSL concentration still positively influences enzyme production, indicating that further increase in this factor may still increase inulinase yield.

The only study that investigated inulinase production by SSF is reported by Selvakumar et al. *(11)*. They obtained about 108 U/g using *Staphylococus* spp. and 123 U/g with *K. marxianus* ATCC 52466 *(11)*. These results are nearly fourfold lower when compared with inulinase activities

Table 4
Matrix of the Second Experimental Design With Coded Levels and Real Values (in Parenthesis) for the Inulinase Activity

Run	Moisture (%)	CSL (%)	Temperature (°C)	Activity (U/g)
1	–1 (50)	–1 (10)	–1 (35)	42.8
2	+1 (80)	–1 (10)	–1 (35)	105.6
3	–1 (50)	+1 (20)	–1 (35)	136.3
4	+1 (80)	+1 (20)	–1 (35)	217.9
5	–1 (50)	–1 (10)	+1 (45)	n.d.
6	+1 (80)	–1 (10)	+1 (45)	11.2
7	–1 (50)	+1 (20)	+1 (45)	11.9
8	+1 (80)	+1 (20)	+1 (45)	52.2
9	–1.68 (39.8)	0 (15)	0 (40)	58.6
10	+1.68 (90.2)	0 (15)	0 (40)	168.5
11	0 (65)	–1.68 (6.6)	0 (40)	90.2
12	0 (65)	+1.68 (23.4)	0 (40)	444.8
13	0 (65)	0 (15)	–1.68 (31.6)	343.4
14	0 (65)	0 (15)	+1.68 (48.4)	n.d.
15	0 (15)	0 (40)	0 (40)	223.3
16	0 (15)	0 (40)	0 (40)	196.1
17	0 (15)	0 (40)	0 (40)	265.7

n.d., not detected.

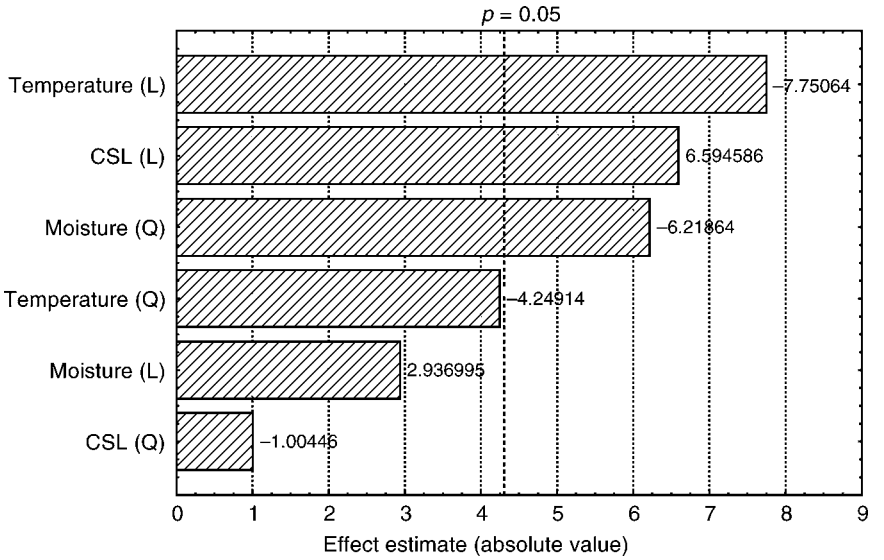

Fig. 2. Pareto chart of effects for the second experimental design.

obtained in our study. This suggests that sugar cane bagasse and CSL are good nutrient sources for inulinase production by *K. marxianus* NRRL Y-7571 using SSF. The moisture content and temperature of incubation that maximize inulinase production were 65% and 40°C, respectively,

similar to those reported by Selvakumar et al. *(11)* where maximum inulinase production by *K. marxianus* was obtained at a moisture content of 65% and temperature of 37°C. A related study of product kinetics was carried out by our group and is described elsewhere *(17)*. Characterization of the inulinase produced by SSF in relation to specificity (endo or exoinulinase), optimum temperature, pH, and stability is currently under investigation.

Other studies concerning inulinase production by *Kluyveromyces* in submerged fermentations show that the amount of enzyme produced greatly vary with the strain and the kind of nutrient supplementation used. Gupta et al. *(18)* obtained maximum production of 7 U·m/L, using fructanes as carbon source for *K. fragilis*. Santos et al. *(10)* reported a production of 7.5 U/g using peptone, sucrose, and yeast extract as nutrient sources for *K. bulgarius*. Kalil et al. *(19)* used sucrose as carbon source and obtained 127 U·m/L of inulinase by *K. marxianus*. In a preliminary comparison, the results obtained in the present work show that production of inulinase by SSF may yield higher amounts of the enzyme. Economical feasibility of inulinase production by SSF is also evident, owing to the use of low cost substrates. An important factor that turns SSF practical for industrial purposes is the simple recovery and purification of the enzyme, because it is found concentrated in the extraction buffer *(12)*.

Conclusions

In this work the maximum inulinase production was 445 U·(g/dry substrate), which is almost fourfold higher than inulinase activities found in literature. The maximum activity was obtained by fermentation of sugar cane bagasse with 65% moisture at 40°C and supplemented with 23.4% of CSL. The results show the feasibility of using SSF for inulinase production by *K. marxianus* and suggest new experimental runs for optimization of enzyme production.

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