FRUCTOSE PRODUCTION FROM DAHLIA TUBER INULIN BY IMMOBILIZED WHOLE YEAST CELLS IN A PACKED BED BIOREACTOR

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ABSTRACT.At present, fructose is commonly produced from starch by the multi - step processes including hydrolysis into glucose followed by the treatment with isomerase and the chromatographic separation of fructose and glucose. In the present work, an alternative process for producing fructose from inulin was investigated. An immobilized biocatalyst with inulinase activity prepared by immobilization of non growing whole yeast cells was tested in a packed-bed bioreactor for continuous fructose production from dahlia tuber inulin. The free whole cells was used for batch inulin conversion in a 2.0 L stirred reactor at substrate concentration of 25-200 $g.L^{-1}$, pH 5.0 and temperature 50 °C for 10 h. The maximum conversion of 72.4 % was obtained from a cultivation containing 125 $g.L^{-1}$ substrate after 6 h. In the second study, the whole yeast cells was immobilized in calcium alginate beads and used for the production at 50 °C and pH 5.0. The optimal gel concentration and cell load were determined The dilution rate of inulin solution (125 $g.L^{-1}$) was varied from 0.08 to 0.30 h^{-1}. The reactor performance was found to be highest at a dilution rate of 0. 20 h^{-1}. In this process, the yield of 109.12 $g.L^{-1}$ fructose, conversion degree of 87.3 % and reactor productivity of 21.83 $g.L^{-1}.h^{-1}$ were obtained, respectively.This bioreactor productivity is noticeably higher than that previously reported. The continuous process was then operated a period of 200 h. However, a good stability of process was achieved during the first 100 h

NTRODUCTION

Fructose syrups are widely used in the food industry. It is an interesting sweetener because of its high sweetening power. Moreover, fructose is claimed to be less cariogenic than other sugars and to be more suitable for diabetics since its metabolism is insulin-non-dependent (Barker and Petch , 1985). Pure fructose, obtained by crystallizing from very high fructose syrups, is used in the pharmaceutical industry as an intravernous nutrient, in the production of the low calorie sweetener (Linden, 1994), and as a sugar source for the production of biosurfactant : fructose-esters (Seino and Uchibori,1994)

At present, fructose is commonly produced from starch by the multi - step processes including hydrolysis into glucose followed by the treatment with isomerase and the chromatographic separation of fructose and glucose., or by the isomerization of a corn glucose syrups. From these process, syrups containing 42% of glucose was obtained. Their fructose content can be increased up to 95% by chromatographic separation and subsequent recycling of the glucose component.

Because of their high content of fructose, inulin is interesting substrate from which to produce fructose (Vandamme and Derycke, 1983). Inulin is composed of β (2-1) linked d- fructose residues, forming linear chains containing one terminal glucose residue on α (1-2) fructose chain (Groot- Wassink and Fleming , 1980). . Inulin as a reserve carbohydrate, found in the roots and tubers of various plants of the family Compositae and Asteraceae, like Jerusalem artichoke, chicory, dahlia, and dandelion (Vandamme and Derycke, 1983). For several reasons, there is strong interest in tropical regions, like

Indonesia or Malaysia in growing dahlia than the others three crops. Many dahlia cultivars are available and have been easily propagated and cultivated yielding satisfactory tubers. Futhermore, dahlia has no common diseases. Dahlia, therefore, can be considered as a promising inulin crops in tropical regions. (Anonymous,2001). Although inulin is readibly hydrolyzed by acid, pH 1 – 2 at 80- 100 °C, there are disadvantages on their use : undesirable colored and flavored materials in the form of anhydride. Enzymatic hydrolysis, therefore, may be the solution as it has no such problems (Barthomeuf *et al*, 1991;Mangunwidjaja, 1997).

Inulin hydrolyzing enzyme - inulinase (2,1 β-D - fructanohydrolase EC 3.2.1.7) have been extracted from roots and tubers of plants of family Asteraceae (Rutherford and Deacon, 1972), and from various fungi (Xiao *et al*, 1989; Barthomeuf *et al*, 1991) bacteria (Allais *et al*, 1986,1987), and yeasts (Margaritis and Bajpai, 1982). Several yeast species, notably *Kluyveromyces fragilis* and *K. marxianus* have been reported as potential inulinase producer. (Duvniak *et al*, 1981)..

We have examined processes for the preparation of high fructose syrups from the dahlia inulin by using commercial inulinase enzyme (Novozym 230) and crude inulinase extract from *Kluyveromyces* culture broth. (Sunarti, *et al* 1994, Mangunwidjaja *et al*,1994). Several studies have been elaborated to produce high fructose syrups from Jerusalem arthicoke by yeasts fermentation (Giraud and Galzy,1990; Fontana, *et al* 1994)

Although it has been showed that the enzyme biocatalyst are adequate from the technical point of view, their cost is high mainly due to the purification and pp cost. In order to reduce their cost, the purification may be avoided and the immobilization procedure simplified using whole cells (Gomez *et al*, 1994). Immobilization of enzyme on whole-microbial cells or nongrowing microbial cells represents in effective way of producing highly different enzyme catalysts applicable in many process of great industrial significance (Hasal, *et al*, 1992a,b)

In this paper, the intracellular inulinase activity of *Kluyveromyces marxianus* whole cells was investigated, and utilized for the production of ultra high fructose syrups from dahlia tuber inulin in a packed-bed bioreactor.

MATERIALS AND METHODS

Chemicals

Inulin, D-glucose, D-fructose used were of analytical grade and purchased from Sigma, Co. Other chemicals were of reagent grade and were purchased from local sources. Alginate (Sigma) used was a medium viscosity grade. Enzyme stock (Novozyme 230) was obtained from NOVO Indonesia branch (Jakarta, Indonesia) Dahlia (*Dahlia pinnata Cav*) inulin was extracted from tubers by hot water extraction and sedimented to % dry matter using the method described by Rahayuningsih (1995) with modifications.

Microorganism and growth conditions

Kluyveromyces marxianus was obtained from Laboratoire de Biochimie microbienne, Universite Henri Poincare, Nancy, France. The medium for the cell cultivation was composed of (g.L^{-1}) : Dahlia inulin 20; bacto peptone 10; Medium without inulin was autoclaved at 105 °C for 30 menit. Inulin was sterilized separately and was added to the medium. The pH was adjusted to 7.0 prior sterilization. Pre-inoculum was prepared from the slant stock by inoculating 100 ml medium in 500 ml Erlenmeyer flask shaker at 300 rpm, 30°C, for 10-15 h . The cultivation was carried out in a 2.0 L bioreactor (Biostat M- Braun, Germany) with working volume of 1.5 L . The temperature was maintained at 30 °C with a thermostat ; aeration and agitation were controlled at 1.0 vvm and 300 rpm. Culture growth was monitored by determining the dry weight of the biomass. Samples were taken at 8 h intervals for enzyme assays. The culture was harvested at the end of exponential growth phase (60 h) and used for immobilization.

Inulinase assay

The intracellular inulinase activity was determined by the method developed by Groot-Wassink dan Fleming (1980) with a modification (Mangunwidjaja, 1997). The 0.5 mL samples taken for enzyme activity measurements were centrifuged immediately at 5,000 g at 5 ° C for 5 min., and the pellets were washed twice with and then suspended in 0.5 ml of 0.1 M sodium acetate buffer solution at pH 5.0 Samples (0.5 mL) and 0.05 µl of 0.02 % fresh lyzozyme solution were homogenized and then incubated at a shaker flask (200 rpm) at 35°C for 4 h. The slurries were further centriguged at 5,000 g (4°C) for 5 min. The pellets were suspended in 0.5 ml of 0.1 M acetete buffer, and were treated as intracellular samples and used for inulinase assay. The reaction mixture (0.1 mL) containing the enzyme samples and 1 % inulin in 0.5 mL of 0.5 M acetate buffer (pH 5.0) was incubated at 50°C for 30 min. The total reducing sugar released was determined by 3,5 dinitrosalicylic acid method (Miller, 1959) using fructose as a standard . One unit of inulinase activity was calculated as 1.0 µ mol of fructose produced by 1.0 g. min^{-1} under the assay condition.

Preparation of cell immobilization

The immobilization technique used was entrapment. The cell pellet obtained after harvesting was resuspended in 0.1 M acetate buffer (pH 5.0). An equal amount of cell suspension of known weight of cells and sodium alginate solution (2.0 %) prepared using the same buffer were mixed with slow agitation. The formation of gel beads was obtained by replacing the sodium ions with calcium ions (1.0 %). Different cell and bead ratios was prepared from 0.05 to 0.25 by varying cell concentration (Mangunwidjaja *et al*, 1994 with modification) The beads were allowed to gel in the calcium chloride solution for 30 min. and then dried on a paper towel for 2 h at room temperature. The alginate beads were then stored at 4°C.

Packed Bed Bioreactor Design

The continuous fructose production was performed in a packed bed bioreactor which was 410 mm tall, 60 mm internal diameter, and had a working volume of 850 ml (Figure 1) The sterilized medium was introduced to the column containing biocatalyst gel beads via a peristaltic pump.The working temperature was maintained at 50 °C by circulating water from a thermostat through a column jacket. The effluent was collected from the outlet pipe in a vessel and prepared for inulin and fructose determination.

Batch Process

The batch process was conducted in a 2.0 L stirred bioreactor (with working volume of 1.50 L) (Biostat-Braun, Germany). The pH and temperature were adjusted to to 5.0 with H_2SO_4, and to 50°C , respectively. To 1.2 L of inulin solution (varied from 25 to 200 g.L^{-1}) in the bioreactor were added 4% (w/v) of biocatalyst.

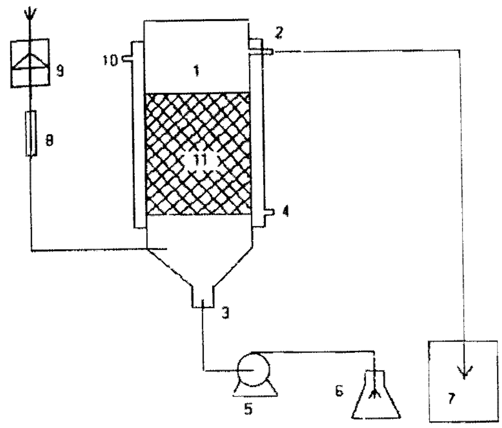

Figure 1: Schematic diagram of the packed bed bioreactor for the production of fructose from inulin using whole celss of *Kluyveromyces marxianus* (1) column bioreactor, (2) outlet, (3) inlet, (4) inlet for jacket water circulation, (5) peristaltic pump, (6) substrate, (7) product collector,(8)air filter, (9)rotameter, (10) outlet for water circulation, (11) packed-bed biocatalyst

Analytical Methods

The biomass was determined by the dry weight technique. Residual inulin in culture broth was determined by using the method of Kiersan (1978) . Fructose was determined as resducing sugars following 3,5 dinitro salicylic acid hydrolysis (Miller, 1959). Protein was estimated according to Lowry et al, (1951) using bovine serum albumin as a standard .

RESULTS AND DISCUSSION

Yeast Growth and Intracellular Inulinase Activity

From the previous works, it was observed that *K. fragilis* and *K. marxianus* among strains were consistently highest inulinase producers (Groot Wassink dan Fleming, 1980; Mangunwidjaja *et al.*, 1994, Sunarti *et al.*, 1994). To obtain a bicatalyst having high intracellular inulinase, the intracellular inulinase activity of *K.marxianus* was investigated during the growth. In our previous studies, we noticed that during 48 h *K.fragilis* cultivation on either synthetic medium (10 g.L^{-1} glucose) or inulin medium, the extracellular inulinase activity was detected in early growth phase and increased related with the biomass (i.e growth-associated) and reached a maximum after 10 h or exponential growth phase. Meanwhile, the intracellular inulinase activity could be observed in the early stationary phase of growth which increased in time and achieved a maximum at the stationary phase (Mangunwidjaja, 1999).

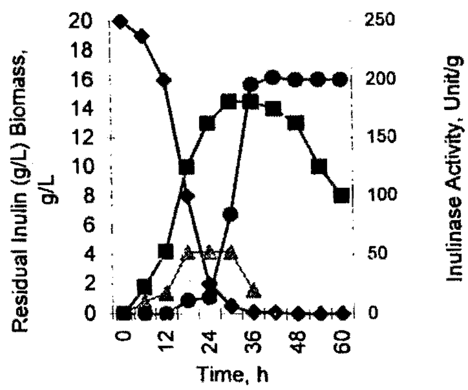

Figure 2: Growth and inulinase production by *K. marxianus* on inulin medium(20 g.L^{-1}) (⊇ residual inulin, ⊂ biomass, σ inulinase activity in culture broth ● intracellular inulinase activity)

The intracellular and extracellular activity of *K.marxianus* observed during 60 h cultures showed that the cells incubated at 50°C have the highest value of 200 Unit.g^{-1} in 12 h and Unit.g-1 in 36 h, respectively (Figure 2)

The result confirmed the possibility of using whole cells containing intracellular inulinase activity for hydrolyzing inulin to fructose. The immobilization conditions and procedures for *K.marxianus* whole cells have been described in our previous work (Mangunwidjaja, 1999), showed that the highest activity was obtained at the highest cell load. However at the cell – bead ratio higher than 0.15 or cell concentration of 6.0 g.L^{-1} inulinase activity was reduced. In addition, the maximum inulinase activity was obtained when cells were entrapped in 2% (w/v) calcium alginate at 50°C, and pH 45- 50 °C.

Therefore, in the present work, the experiments were carried out using the biocatalyst composing 6.0 dry biomass, L^{-1} gel, at 50°C and pH 5.0

Fructose Production in Batch Bioreactor

The immobilized cells prepared under optimal conditions were then used to hydrolize dahlia inulin solution. Several experiments were carried out at 50°C, pH 5.0 and a agitation rate of 60 rpm. The initial substrate concentration was varied from 25 to 200 gL^{-1}. The inulin conversion rate was determined by measuring the amount of fructose produced by 4% (w/v) of biocatalyst in 100 mL of dahlia inulin solution after 6 h. As shown in Figure 3 the highest conversion rate of 17.4 and 15.08 g $fructose.L^{-1}.h^{-1}$ was obtained, by using free and immobilized biocatalysts, respectively from substrate at concentration 125 – 150 gL^{-1}. For the substrate concentration higher than 150 $g.L^{-1}$ the conversion rate decreased to 9.2 and 4.1 g $fructose.L^{-1}.h^{-1}$.

Based on the above results, a batch process was then conducted in a 2.0 L stirred bioreactor (with 1.5 L working volume). The substrate and biocatalyst concentrations were 125 gL^{-1} and 4% (w/v), respectively. The amount of inulin was converted predominantly during the initial state of the reaction, and reached maximum after 6 h. In this process, the yield of 90.5 $g.L^{-1}$ fructose or 72.4 % conversion was obtained. The bioreactor productivity was calculated to be 15.08 $g.L^{-1}.h^{-1}$.

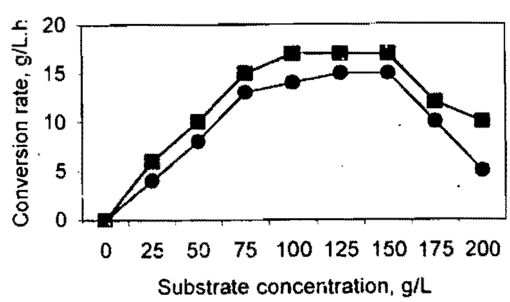

Figure 3: Conversion rate of dahlia inulin to fructose at 50 ° C, pH 5,0 on a various substrate concentration (■ free biocatalyst ● - immobilized biocatalyst)

The present work offers a significant improvement over those previously reported using immobilized cells of *K. fragilis* ATCC 12424 (Mangunwidjaja *et al*, 1994), free crude inulinase enzyme of *Penicillium regulosum* (Berthemeuf *et al*,1991).However, the results was lower than those obtained by Baratti and Ettalibi (1994) resulting 99-100% inulin conversion from a continuous fixed-bed reactor.

Continuous Fructose Production in Packed Bed Bioreactor

The utlization of biocatalyst for hydrolysis process in batch reactor usually presents drawbacks, among others, long reaction time, large reactor volume, poor enzyme stability (Klein *et al*, 1985, Santoyo *et al*, 1998). Considering for process control during the reaction, the deactivating effect of the biocatalyst, and the simplicity of the bioreactor design, a packed-bed bioreactor was proposed for performing a continuous process (Mangunwidjaja *et al*, 1997)

The continuous production of fructose from dahlia inulin solution using immobilized non growing *K.marxianus* whole cells was conducted in a 1.0 L packed-bed bioreactor (with working volume 0.850 L)- Figure 1., at 50°C and pH 5.0. The feed flow rate of inulin solution (125 g/L) was varied from 0.068 to 0.255 Lh^{-1}. At steady state condition achieved after 3-6 h operation, the production of fructose was determined. .

Table 1: Fructose yield, inulin conversion and bioreactor productivity at different substrate flow rate on hydrolysis of dahlia inulin (125 $g.L^{-1}$) in a packed-bed bioreactor .

Substrate flow rate ($L.h^{-1}$)	Fructose yield ($g.L^{-1}$)	Inulin conversion (%)	Bioreactor productivity ($g.L.h^{-1}$)
0.08	28.12	22.5	5.62
0.12	30.00	34.0	6.00
0.16	80.00	62.4	16.00
0.20	109.13	87.3	21.83
0.24	51.50	41.2	10.30
0.30	48.75	39.0	9.75

The inulin conversion and bioreactor productivity as a function of substrate flow rate is presented in Table 1, and it can observed that the conversion increased with increase in substrate flowrate. The highest conversion of 87.3% was obtained at a flow rate of 0.170 $L.h^{-1}$. Based on the results, the bioreactor was then run for longer time to test the biocatalyst stability.

Operational Stability

The stability of the inulinase activity of the biocatalyst was examined in a continuous packed-bed biorator at constant substrate flowrate (0.170 $L.h^{-1}$), at 50°C, pH 5.0 and constant inlet inulin concentration (125 $g.L^{-1}$). The total duration of the trial was 200 h. The reasult is presented in Figure 4.

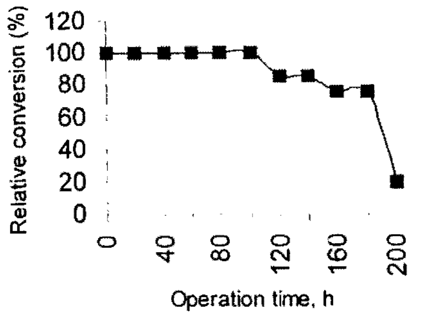

Figure 4: Stability of continuous process of packed-bed bioreactor using for fructose production from dahlia inulin (125 $g.L^{-1}$)

The initial value of the conversion was 87% . The result indicated that a good stability of continuous operation was achieved during the first 100 h. After that time, the inulin conversion decreased drastically with increasing operation time to 8.2 %. The bioreactor productivity has also reduced from 21.83 to 2.05 g fructose.$L^{-1}h^{-1}$.

The drastic reduction of biocatalyst activity probably due to the thermal inactivation of the enzyme because cell must be maintained at high temperature (50 °C) for long run operation, and due to the mechanical abrasion of beads (Kim *et al* 1982). Therefore, alternatives immobilizing gels like carragenan, chitosan are proposed. In addition, stabilizing treatments of the immobilized whole cells preparation by covering the beads with hardening reagents, i.e. glutaraldehyde, hexamethylenediamine, and polyethelene are also considered. (Santoyo *et al*, 1998, Dobreva *et al*, 1998).

CONCLUSION

The experiments summarized in this paper showed that the biocatalyst with inulinase activity prepared by immobilization of whole cells of *K. marxianus* can be utilized in a continuous packed-bed bioreactor for the production of fructose from dahlia inulin.

In a continuous process using the biocatalyst, a conversion rate as high as 87 % was achieved at a substrate (125 $g.L^{-1}$) of 0.170 $L.h^{-1}$. Bioreactor productivity was obtained up to 21.83 g fructose.$L^{-1}.h^{-1}$, and a good stability of operation was achieved for a period of 100 – 120 h.

Due to the low cost of the immobilization procedure, production of ultra high fructose syrups from dahlia inulin by means of the biocatalyst promises to be economically very attractive.

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