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Comparative Study on Production of Fructooligosaccharides by *p. Simplicissimum* using Immobilized cells and Conventional Reactor System

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***Abstract*—**Fructooligosaccharides derived from microbial enzyme especially from fungal sources has been received particular attention due to its beneficial effects as prebiotics and mass production. However, fungal fermentation is always cumbersome due to its broth rheology problem that will eventually affect the production of FOS. This study investigated the efficiency of immobilized cell system using rotating fibrous bed bioreactor (RFBB) in producing fructooligosaccharides (FOS). A comparative picture with respect to conventional stirred tank bioreactor (CSTB) and RFBB has been presented. To demonstrate the effect of agitation intensity and aeration rate, a laboratory-scale bioreactor 2.5 L was operated in three phases (high, medium, low) for 48 hours. Agitation speed has a great influence on *P. simplicissimum* fermentation for FOS production, where the volumetric FOS productivity using RFBB is increased with almost 4 fold compared to the FOS productivity in CSTB that only 0.319 g/L/h. Rate of FOS production increased up to 1.2 fold when immobilized cells system was employed at aeration rate similar to the freely suspended cells at 2.0 vvm.

***Keywords*—**fructooligosaccharides, immobilized, productivity, prebiotics

I. Introduction

FRUCTOOLIGOSACCHARIDES (FOS) is a class of oligosaccharides used as an artificial or alternative sweetener. FOS usage emerged in the 1980s in response to consumer demand for healthier and calorie-reduced foods [1]. FOS is known as functional food and has been a popular dietary supplement in Japan for many years [2]. The possible health benefits associated with the consumption of these compounds offer such benefit for reducing lipoprotein cholesterol ratio [3] and body fat content [4] especially for people that is obese. Also, it is suitable for consumption of diabetic patient due to the non-caloric and non-cariogenic as a sweetener. FOS has been produced by the action of transfrutosylation activity by the enzyme fructosyltransferase from many plants and microorganisms. FOS is common to edible parts of a variety plants like onion, Jerusalem artichoke, chicory roots, leek, garlic, banana, rye, yacon, and salsify [1, 5]. However, the production yield of FOS using enzymes originated from plants is low and the mass production of this enzyme is quite limited by seasonal conditions, therefore industrial production depends chiefly on microbial enzymes. FOS has also been commercially produced using fructosyltransferase (FTase) obtained from various microorganisms such as *Aspergillus aculeatus* [6], *Aspergillus foetidus* [7], *Bacillus subtilis* [8], *Bacillus macerans* [9], *Streptococcus salivarius* [10] and *Aureobasidium pullulans* [11]. However, filamentous fungal fermentation is a complex process that affects broth rheology which leads to numerous problems in gas dispersion, mass and heat transfer, and mixing in a conventional stirred tank bioreactor [12, 13]. Therefore, controlling the fungal morphology is required to obtain higher production rate and good performance. Various cell immobilization methods for FOS production are being considered to overcome the problem in fungal fermentation [14, 15, 16]. In the present study, the production of FOS by *Penicillium simplicissimum* using immobilized and freely suspended cells was compared based on agitation speed and aeration rate effects.

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II. Materials And Methods

A. Microorganism and culture media

The fungus, *Penicillium simplicissimum,* a local isolate was obtained from Department of Botany, Institute of Biological Science, University of Malaya, Kuala Lumpur. The strain was maintained by weekly transfer on potato dextrose agar (PDA) and stored at 4^{o}C after incubated at 33^{o}C for 5 day. Monthly subculture ensured the availability of sufficient stock cultures for experimental processes. The composition of the production medium consisting the following (g/L): freeze dried sugarcane juice 300, KH_2PO_4 11, NH_4Cl 6, and yeast extract 10. The medium was then sterilized at 121^{o}C for 30 min. The inoculum for the bioreactor (15 %v/v) was prepared from the mycelia mats of stock culture. Five round disks of 0.50 cm were punched on the mycelia mats by a sterile cork borer. The disks were then put into an Erlenmeyer flask containing 100 ml growth medium (1%(w/v) sucrose and 0.2%(w/v) yeast extract at pH 5.5). The cultures were incubated in a rotary shaker (Innova 40, New Brunswick Scientific) at 33 ± 1 ˚C, 250 rpm for 24 h.

B. Experimental setup

Fermentations were performed in a 2.5 L bioreactor (Minifors, Infors AG, Switzerland) using a working volume of 1.5 L. Agitation was provided by two 6-bladed Rushton impellers of diameter 4.5 cm. The vessel possessed 4 vertical baffles, temperature probe (PT-100), pH, pO_2, and antifoam probes, harvesting and gas supply pipes with ring sparger, exhaust cooler and 3 storage bottles for acid, alkaline, and

antifoam. A microprocessor system (IRIS software) capable of PID (Proportional Integral Derivative) control of temperature (±1 ˚C), agitation speed (±1 rpm), pH (±0.01), and dissolved oxygen (DO) (±1%) were also employed. For immobilized cells system, the 2.5-L bioreactor was modified by affixing a perforated stainless steel cylinder mounted with a fibrous material to the agitation shaft using the method as described by Tay and Yang [16]. The cylindrical matrix of 9 cm diameter and 15 cm height was used in this study. This was setup to immobilize the fungal spores and mycelia in a rotating fibrous material in a stirred tank bioreactor, so as to control the fungal morphology, oxygen transfer, and separation of cells from the culture broth. Since the fungal cells were immobilized onto a rotational supporting matrix in sterile control conditions of the bioreactor, the bioreactor is known as rotating fibrous bed bioreactor [16]. The bioreactor was set up to the controlled parameters at 33˚C, pH 6.0, 250 rpm, and aerated at 1.0 vvm. The production medium was previously sterilized prior inoculating 15 %(v/v) of inoculums.Freely suspended cells experiments were performed using a working volume of 1.5 L. The production medium was prepared and sterilized at 121˚C for 30 min. After sterilization, the bioreactor was set up to the controlled parameters at 33 ˚C, pH 6.0, 250 rpm, and aeration at 1 vvm, prior inoculated with 15 %(v/v) of inoculum.

C. Determination of biomass

The cultures were harvested at designed intervals, centrifuged (4000 x g 20 min, 4˚C) and filtered using filter paper (Whatman No. 2) to separate the pellets from the culture broth, in which the supernatant was later used as an enzyme source, fructosyltransferase (FTase). The pellets were washed with distilled water several times. The biomass was determined using dry cell weight (DCW) by drying the pellets to constant weight at 70 ˚C for 24 h in an oven (Memmert, Model UNB 500yu). The biomass produced was expressed as g/L.

D. Crude enzyme preparation

The extracellular enzyme source was obtained from the supernatant and was used without any pretreatment. The intracellular enzyme source was prepared as follows. The culture broth containing cells and culture fluid was sonicated together at 4˚ C, 20 kHz for 15 min. The homogenate obtained was then centrifuged at 4˚ C, 4500 rpm for 20 min to separate cells and supernatant. The supernatant obtained was used as intracellular enzyme source.

E. FOS production

FOS production was determined by incubating 1.5 mL of 60% (w/v) sucrose in 0.5 mL of 0.1 M citrate buffer (pH 5.5) and 0.5 mL crude enzyme-culture fluid at 55±1 ˚C for 1 to 36 h. The reaction mixture was terminated by dipping the tubes in boiling water bath for 15 min [17]. The final FOS yield (g/g) is expressed based on initial sucrose concentration.

F. Analytical procedures

The FOS concentration was determined using a high performance liquid chromatography (HPLC) (LC-10 A, Shimadzu, Japan) with a refractive index detector and polar bonded phase column (Supercosil LC-NH_2, 4.6 mm x 25 cm, 5 µm) at room temperature (~ 24^{o}C). The flow rate of the mobile phase (acetonitrile: water (75:25)) was 1.0 mL/min [17]. The retention times of the individual FOS were compared with the reliable standards of 1-kestose, 1-nystose and fructofuranosyl nystose for identification.

III. RESULTS AND DISCUSSION

The comparison of the mycelial cell growth and FOS production by *P. simplicissimum* cultivated in the conventional stirred tank bioreactor (CSTB) and rotating fibrous bed bioreactor (RFBB) are summarized in Table I with two parameters which are agitation speed and aeration rate ranging at low, medium, and high measurements, respectively.

TABLE I
EFFECT OF AGITATION SPEED ON PRODUCTION OF FOS USING FREELY SUSPENDED AND IMMOBILIZED CELL SYSTEM

Parameter	100 rpm	150 rpm	300 rpm
Freely suspended cell in CSTB			
Final cell biomass, X_f (g/L)	2.208	4.582	8.964
Maximum FOS yield (g/g)	0.255	0.433	0.666
Volumetric cell biomass productivity (g/L/h)	0.046	0.095	0.187
Volumetric FOS productivity (g/L/h)	0.319	0.541	0.833
Specific growth rate, μ_0 (1/h)	0.039	0.060	0.083
Doubling time, t_d (h)	17.773	11.552	8.351
Immobilized cell in RFBB			
Final cell biomass, X_f (g/L)	9.964	7.023	2.114
Maximum FOS yield (g/g)	1.053	1.651	0.163
Volumetric cell biomass productivity (g/L/h)	0.208	0.146	0.044
Volumetric FOS productivity (g/L/h)	1.316	2.064	0.204
Specific growth rate, μ_0 (1/h)	0.085	0.077	0.058
Doubling time, t_d (h)	8.154	9.002	11.95

The production of FOS was scale up in a 2.5 L bioreactor with working volume 1.5 L using both freely suspended and immobilized cells system incubated at 33 ˚C in CSTB and RFBB, accordingly. The samples for both systems were withdrawn every 4 h interval. For the immobilized cells system in the RFBB the biomass was harvested either every 4 hour or at the end of the fermentation period. And those that were suspended or detached from the supporting matrix or attached onto the matrix at 48 h, so called overall or final cell biomass, X_f were considered. The cells sample that was withdrawn every 4 h interval in immobilized cell system was correlated with the final cell biomass, X_f to obtain the cells concentration that adsorbed onto the fibrous surface as shown in Fig. 1 and Fig. 2 of Appendix.

Table I compares the effect of different agitation speed (100, 200, and 300rpm) on mycelial growth and FOS production by *P. simplicissimum* using sugarcane juice as a

growth medium in a bioreactor using freely suspended and immobilized cell systems. As one can observed, in freely suspended cells system, the biomass and specific growth rate were high (8.964 g/L, 0.072 1/h) at agitation rate 300 rpm, while for immobilized cell system the biomass attached onto the fibrous matrix and the specific growth rate were high (9.964 g/L, 0.085 1/h) when the agitation was kept at 100 rpm (Table I). Thus, it is showing that the agitation speed should be slower so that more cells could be attached or immobilized onto the fibrous material. At high agitation speed (300 rpm), only a few cells were attached onto the surface of the fibrous material (2.114 g/L) and the culture broth was full with filamentous cells that made the medium cloudy.

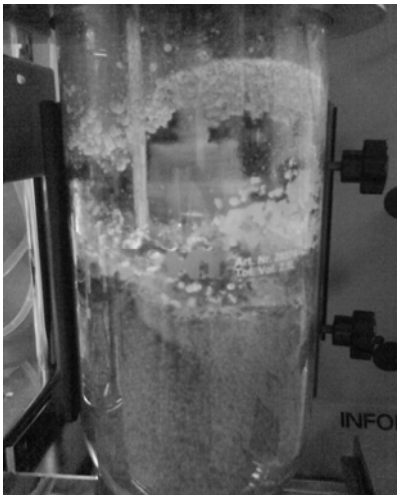

Fig. 3 Medium was filled with pellets and the filamentous fungus of freely suspended cells attached everywhere

As can be seen in Fig. 3, not only numerous mycelial clumps (loose pellets) and small fragments of mycelium in suspension, large clumps of mycelia were also grown everywhere in the CSTB. They attached to the sparger, probes, and culture vessel wall, hindering the control of fermentation conditions at the setting variables. These resulted in the difficulty in mixing, which in turn affected mass and heat transfer, thus causing the decreased in productivity and the increase in the production of undesirable metabolites [18]. According to Thongchul [19], the strong mechanical forces could deactivate loose mycelia at some level of magnitude. Due to that, the mycelial biomass at the end of the fermentation (48 h) was only about 38.5% of those attached cells obtained in RFBB at 300 rpm. Thus confirming that RFBB was superior in supporting cell growth by immobilizing (adsorption) the mycelia onto the supporting matrix attached onto the rotating shaft of the bioreactor at slower agitation speed (100 rpm). According to Xu and Yang [13], RFBB provided the advantage of controlling mycelial growth and morphology of *P. brevicompactum* during the fermentation of mycophenolic acid.

Fig. 4 Attachment of *P. simplicissimum* mycelium seen in the RFBB at the end of the fermentation process

Fig. 4 shows that almost no mycelia was found suspended in the culture broth, instead forming clean, clear and non-viscous like water, which facilitated the control and operation of RFBB during the fermentation. Similar observations were also reported by several researchers using *Rhizopus oryzae*, *Xanthomonas campestris*, *Lactobacillus helveticus*, and *Clostridium tyrobutyricum* as a tested fungus in the RFBB [16, 20, 21, 22, 23, 24].In freely suspended fermentation in CSTB, the *P. simplicissimum* cells grew in the form of pellets. Many hyphae were bound in the outer zone of the round pellets. It was found that higher the concentration of sucrose in the production media, larger was the pellets. These results are in accordance with the filamentous form of mycelia of *P. simplicissimum*. However, these pellets become fragmented due to higher agitation and aeration rates. According to Cui [25], when substrate and oxygen were sufficient, the breakage of pellets occurred less often. Further increase in agitation intensity did not increase FOS production.From Table II results shows that further increase in aeration rate resulted in a greater FOS production for both systems. FOS production increased up to 1.2-fold when the immobilized cells system was employed at aeration rate similar to the freely suspended cells at 2.0 vvm. The highest specific growth rate was observed at 0.085 1/h with the shortest doubling time (8.154 h) when 100 rpm of agitation speed was employed for the immobilized cells system. Agitation speed has a great influence on *P. simplicissimum* fermentation for FOS production, especially in immobilized cell system. Also, the highest FOS yield (1.651 g/g) was observed when 150 rpm of agitation speed was employed in the culture broth using immobilized cell system. Thus indicating that the immobilized cells in rotating fibrous bed bioreactor was found to be superior to the freely suspended cells fermented in conventional stirred tank bioreactor.

TABLE II
EFFECT OF AERATION RATE ON PRODUCTION OF FOS USING FREELY SUSPENDED AND IMMOBILIZED CELL SYSTEM

Parameter	0.5 vvm	1.0 vvm	2.0 vvm
Freely suspended cell in CSTB			
Final cell biomass, X_f (g/L)	2.254	3.559	7.348
Maximum FOS yield (g/g)	0.587	0.739	0.756
Volumetric cell biomass productivity (g/L/h)	0.047	0.074	0.153
Volumetric FOS productivity (g/L/h)	0.734	0.924	0.945
Specific growth rate, μ_0 (1/h)	0.037	0.046	0.049
Doubling time, t_d (h)	18.733	15.068	14.146
Immobilized cell in RFBB			
Final cell biomass, X_f (g/L)	3.564	5.725	6.873
Maximum FOS yield (g/g)	0.714	0.802	0.812
Volumetric cell biomass productivity (g/L/h)	0.074	0.119	0.143
Volumetric FOS productivity (g/L/h)	0.893	1.003	1.015
Specific growth rate, μ_0 (1/h)	0.048	0.060	0.061
Doubling time, t_d (h)	14.441	11.552	11.363

IV. CONCLUSION

As a conclusion immobilized cells bioreactor is better than free culture bioreactor in FOS production. Rotating fibrous bed bioreactor is a novel and efficient one, which can be adopted for FOS production using filamentous fungi where agitation speed showed pronounced effect in immobilized cell system as it determined the mass transfer in the system through a better diffusion of sugars, other nutrients, and oxygen supply to the cells.

APPENDIX

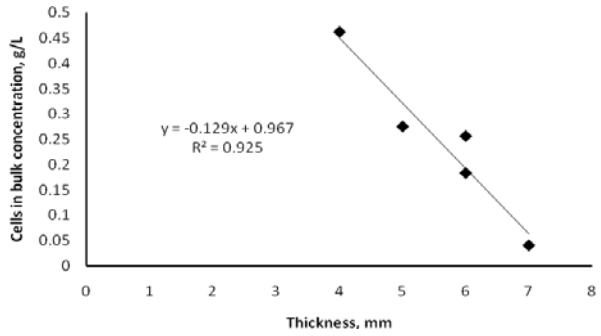

Fig. 1 Correlation between the thickness of biofilm and cells concentration in bulk (freely suspended cells in the broth culture)

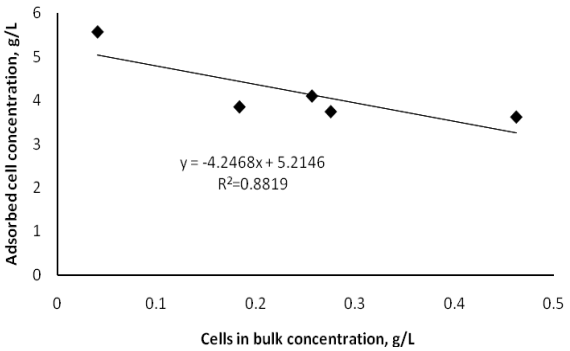

Fig. 2 Correlation between the immobilized cell concentration onto the fibrous matrix and cells concentration in bulk (freely suspended)

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