J. Microbiol. Biotechnol. (2005), **15**(6), 1317–1322

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Immobilization of *Penicillium citrinum* by Entrapping Cells in Calcium Alginate for the Production of Neo-Fructooligosaccharides

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Received: March 29, 2005
Accepted: Mary 16, 2005

Abstract This work describes neo-fructooligosaccharides (neo-FOSs) production using the immobilized mycelia of *Penicillium citrinum*. Some critical factors were evaluated to optimize maximal production of neo-FOS. Optimal alginate and cell concentrations were determined to be 1.96% alginate and 7.17% cell, respectively, by statistical analysis. The optimal concentration of $CaCl_2$, which is related to bead stability, was determined to be 2 M. It was possible to increase the neo-FOS production by adding 15 units of glucose oxidase to the batch reaction. By co-immobilizing cells and glucose oxidase, neo-FOS productivity increased 123% compared with the whole-cell immobilization process. Based on the results above, a co-immobilization technique was developed and it can be utilized for large-scale production.

Key words: Neo-fructooligosaccharide, *Penicillium citrinum*, calcium alginate immobilization, co-immobilization

Fructooligosaccharides (FOSs) have attracted attention because of an increased demand as so-called health foods. These fructose oligomers are mainly composed of 1-kestose (GF_2), nystose (GF_3), and 1^F-β-fructofuranosyl nystose (GF_4), and can be produced from sucrose by transfructosylating action of enzymes obtained from various microorganisms and plants [1–4]. Similar to lactosucrose, galactooligosaccharides, isomaltooligosaccharides, and glucosylsucrose, FOS has attracted attention because of its abilities to promote the growth of bifidobacteria in the intestinal tract and to decrease the content of putrefactive substances [5, 6]. In addition, FOSs are noncariogenic and noncaloric, but have a sweet taste with 40 to 60% the sweetening power of sugar [8]. The FOS-producing enzymes are usually classified as β-D-fructofuranosidase (invertase, EC. 3.2.1.26) and fructosyltransferase (EC. 2.4.1.9). The former was probably named because its fructosylating activity was originally found during investigations of invertase action on high concentrations of sucrose. The enzymes with potential for industrial application are originated from several fungi, and include *Aureobasidium* sp. [8], *Aureobasidium pullulans* [9], and *Aspergillus niger* [10, 11].

Several studies showed structural differences between neo-fructooligosaccharides (neo-FOSs) produced by microorganisms [12–14]. Neo-FOSs consist mainly of neo-kestose (neo-GF_2) and neo-nystose (neo-GF_3), in which fructosyl units are bound at the β(2–6) position of sucrose. Kilian *et al.* [14] reported that neo-kestose from *Xanthophyllomyces dendrorhous* is a potential novel bifidogenic substance, and that it might have advantages over current commercially available sources.

During the past few years, many industrial FOS production processes have been developed using either immobilized enzymes or cells for FOS production [15–19], but no investigation has been carried out on the neo-FOS production processes using either whole-cell immobilization or co-immobilization of cells and glucose oxidase.

In this study, the mycelia of *Penicillium citrinum*, which produce FOS and neo-FOS, were immobilized in calcium alginate, and initial immobilization conditions were established for an industrial neo-FOS production process.

Materials and Methods

Microorganism and Culture Medium

Penicillium citrinum was obtained from the CJ Corporation. It was subcultured at an interval of two months and stored

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Table 1. Central composite design matrix of independent variables with corresponding experimental and predicted values.

Run	Alginate conc.		Cell conc.		Bead intensity		Neo-FOS production	
	X_1 (%)	Code x_1	X_2 (%)	Code x_2	Experimental	Predicted	Experimental	Predicted
1	1.50	-1	5.00	-1	0.352	0.355	42.25	37.83
2	2.00	1	5.00	-1	0.441	0.446	45.95	44.89
3	1.50	-1	10.00	1	0.252	0.263	31.54	29.34
4	2.00	1	10.00	1	0.379	0.392	41.43	42.58
5	1.40	-1.414	7.50	0	0.339	0.332	30.41	34.41
6	1.75	0	3.97	-1.414	0.373	0.370	36.36	39.56
7	2.10	1.414	7.50	0	0.497	0.488	49.50	48.76
8	1.75	0	11.04	1.414	0.279	0.266	31.86	31.92
9	1.75	0	7.50	0	0.422	0.454	47.88	47.61
10	1.75	0	7.50	0	0.458	0.454	46.84	47.61
11	1.75	0	7.50	0	0.483	0.454	48.12	47.61

on sucrose and yeast extract agar plates at 4°C. Seed and main culture media consisted of 20% (w/v) sucrose, 0.2% (w/v) $NaNO_3$, 0.5% (w/v) K_2HPO_4, 2% (w/v) yeast extract, 0.1% (w/v) $MgSO_4 \cdot 7H_2O$, 0.1% (w/v) KCl, and 0.002% (w/v) $FeSO_4 \cdot 7H_2O$. The pH was adjusted to 6.0 before the medium was sterilized. Seed was incubated at 28°C for 72 h at 200 rpm in a rotary shaking incubator. Seed culture (5%, v/v) was then transferred to the main medium, and the main culture was carried out at 28°C for 90 h at 200 rpm in a rotary shaking incubator. Seed and main cultures were carried out in 500-ml Erlenmeyer flasks containing 100 ml of medium.

Whole-Cell Immobilization

Mycelia of *P. citrinum* were immobilized by entrapment using calcium alginate. Mycelia were mixed with sodium alginate solution, and the mycelia-alginate mixture was then extruded dropwise through a needle (ID 1.0 mm) into a 0.2 M $CaCl_2$ solution using a peristaltic pump. Droplets were instantly transformed into spherical gel beads (approximately 2–2.5 mm in diameter) by spontaneous exchange of Na^+ in droplets and Ca^{2+} in solution. Beads were kept in $CaCl_2$ solution at 4°C for 24 h before use.

Co-Immobilization Using Cell and Glucose Oxidase

One gram of glucose oxidase powder (Novozyme) was dissolved in 100 ml of 0.1 M sodium acetate buffer (pH 5.4). Free enzyme suspension was added to 100 ml of sucrose solution (60%, w/v) containing 10 g of cell-immobilized beads to determine the optimal amount of glucose oxidase required. In the case of co-immobilization, a mixture of mycelia and glucose oxidase activity of 15 units per gram of sucrose was immobilized by entrapment using calcium alginate, as described previosuly [2, 3].

Analysis

Ten grams of cell-immobilized beads were added to 100 ml of sucrose solution (60%, w/v) and allowed to produce neo-FOS at 50°C for 24 h at 100 rpm in a shaking incubator. Reaction products were analyzed by HPLC (Young-Lin Instrument Co. Ltd., Seoul, Korea) using a Daisogel SP-120-5-ODS-BP column (150×6.0 mm, Higgins Analytical, Inc., Mountain View, CA, U.S.A.) and a refractive index detector (Young-Lin Instrument Co. Ltd.). The column temperature was maintained at 50°C, and water was used as a mobile phase at a flow rate of 1.0 ml/min. Total amounts of neo-FOS were the sum of neo-kestose (n-GF_2) and neo-nystose (n-GF_3).

Cell-immobilized bead intensities were analyzed using a texture analyzer. The mean intensity of eight beads was calculated and taken as the bead intensity under each experimental condition.

Experimental Design

A factorial central composite experimental design with four starting points ($\alpha = \pm 1.414$) and three replicates at the center point was employed. The resulting 11 experiments were used to optimize the alginate and cell concentrations. Variables were coded according to the equation:

$$x_i = (X_i - X_0)/\Delta X \quad i = 1, 2, 3 \ldots, j \tag{1}$$

Table 2. Analysis of variance (ANOVA), least-squares fit, and parameter estimates for the bead intensity model.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P>F*
Model	0.062	5	0.012	24.62	0.0016
Error	0.0025	5	0.0005		
Corrected total	0.0641	10			

Coefficient of variation (CV)=5.76%; coefficient of determination (R^2)=0.9610; correlation coefficient (R)=0.9433.

Table 3. Analysis of variance (ANOVA), least-squares fit, and parameter estimates for the neo-FOS production model.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P>F*
Model	482.82	5	96.56	8.86	0.0159
Error	54.51	5	10.90		
Corrected total	537.34	10			

Coefficient of variation (CV)=8.0%; coefficient of determination (R^2)=0.8985; correlation coefficient (R)=0.9433.

where x_i is the coded (dimensionless) value of the variable X_i, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point, and ΔX is the step change.

The behavior of the system was explained by the following second-degree polynomial equation:

$$y=\beta_0+\Sigma\beta_i x_i+\Sigma\beta_{ii}x_i^2+\Sigma\beta_{ij}x_i x_j \quad (2)$$

where y is the predicted response, β_0 is the offset term, β_i is the linear effect, β_{ii} is the squared effect, and β_{ij} is the interaction effect. SAS ver. 8 (SAS Institute Inc., Cary, NC, U.S.A.) was used for the regression analysis of the data obtained and to estimate regression equation coefficients. The significance of regression equation was determined by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Determination of the Optimal Cell and Alginate Concentrations for the Immobilization of *P. citrinum*

To develop a good immobilized cell reactor, the optimal concentrations of immobilized matrix components must be satisfied. In this study, the optimal concentrations of cell and alginate concentrations for neo-FOS production were determined by statistical analysis. To maintain the long-term performance of the bioreactor, the effects of the cell and alginate concentrations on bead intensity were investigated. The experimental and predicted responses of neo-FOS production and bead intensity are given in Table 1. Using multiple regression analysis on the experimental data, the following second-order polynomial equations were found to describe bead intensity and neo-FOS production:

$$\text{Bead intensity}=0.45+0.05x_1-0.02x_1^2 -0.04x_2-0.07x_2^2+0.01x_1x_2 \quad (3)$$

$$\text{Neo-FOS production}=47.61+5.07x_1-3.01x_1^2 -2.70x_2-5.94x_2^2+1.55x_1x_2 \quad (4)$$

where x_1=coded value of the alginate concentration, x_2= coded value of the cell concentration.

Statistical testing of the model was done using Fisher's statistical test for analysis of variance (ANOVA), and the results are shown in Tables 2 and 3. If the model predicts well the experimental data, the computed F-value should be higher than the F-value in the tables. Moreover, the closer the value of R (multiple correlation coefficient) to 1, the better the correlation between the observed and predicted values. In the case of the bead intensity model, R (=0.9803) and R^2 (=0.9610) values indicate that 96.1% of the variability in the response is explained by the model. Parameter estimates and corresponding P values (Table 2)

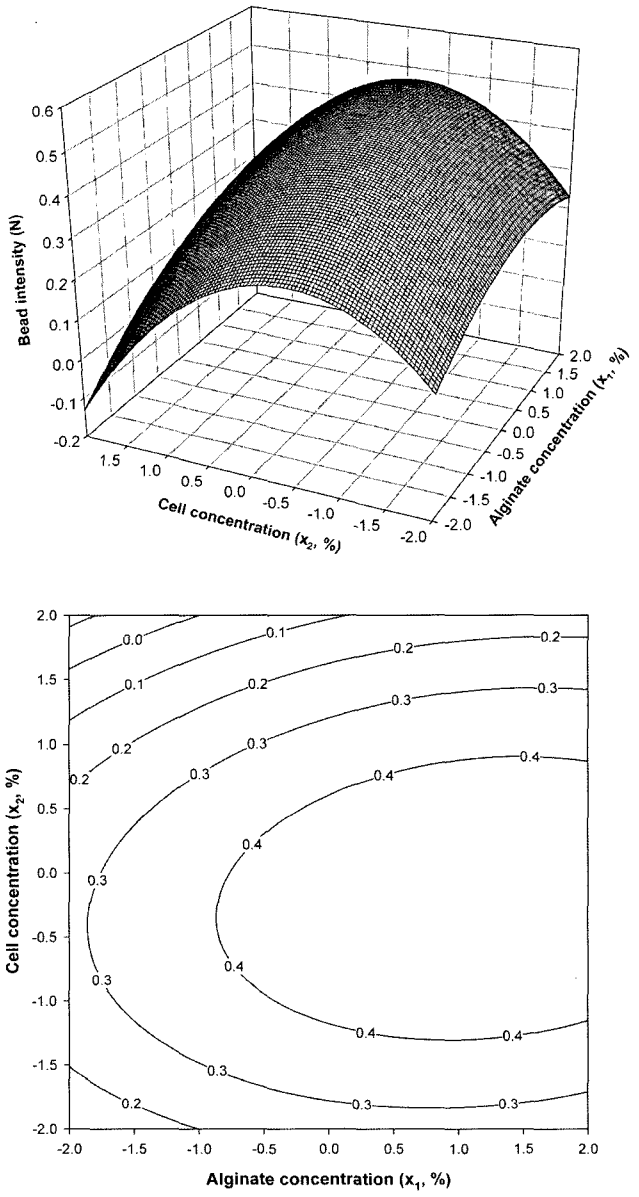

Fig. 1. Isoresponse contour plot showing the effect of alginate and cell concentration on bead intensity.

suggested that alginate concentration had a more positive effect on bead intensity than cell concentration. Response surface plots provided a method for predicting response to different test variable values, and plot contours helped identify the types of interactions between test variables. As shown in Fig. 1, since the contour plots are circular, it can be inferred that interaction between variables is negligible. The optimal points producing maximum bead intensity were 1.96% alginate (x_1=0.848) and 7.17% cell (x_2=−0.132), and the predicted maximum value of bead intensity was 0.491 N. In the case of the neo-FOS production model, R and R^2 values were 0.9479 and 0.8985, respectively. Parameter estimates and corresponding P values (Table 3) suggested that alginate concentration had a more positive effect on neo-FOS production than cell concentration. The response surface plot and the contour plot of this model showed tendencies similar to that of bead intensity (Fig. 2). The optimal points giving maximum neo-FOS production were 1.89% alginate (x_1=0.573), 7.28% cell (x_2=−0.086), and the predicted maximum value of neo-FOS production was 49.83 g/l. However, a small difference was observed between the optimal values of the two models. By substituting these values in Eqs. (3) and (4), it was found that 1.96% alginate and 7.17% cell concentration gave maximum bead intensity and neo-FOS production. Thus, the optimal alginate and cell concentrations for neo-FOS production, using a cell immobilized reaction, were determined to be 1.96% and 7.17%, respectively.

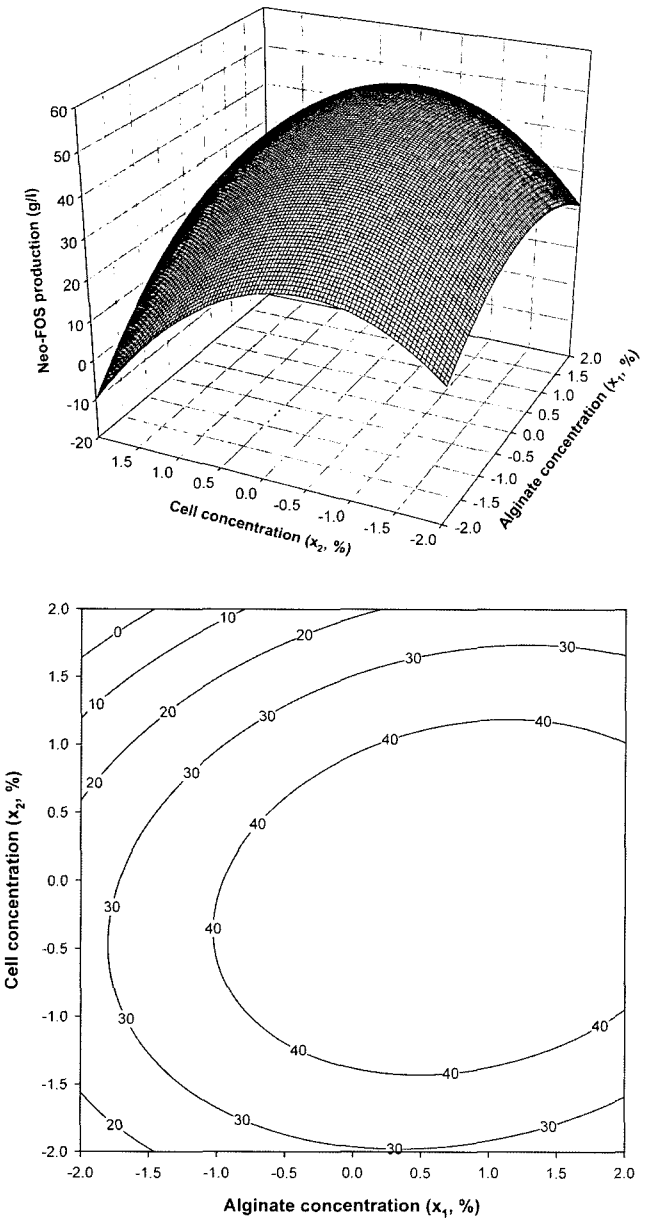

Fig. 2. Isoresponse contour plot showing the effect of alginate and cell concentration on neo-FOS production.

Effect of $CaCl_2$ Concentration on Bead Intensity and Neo-FOS Production

Devi and Sridhar [20] reported that $CaCl_2$ concentration had a profound effect on cephamycin C production, using *Streptomyces clavuligerus* immobilized on alginate beads. Generally, $CaCl_2$ concentration was related to bead intensity and stability. Thus, we investigated the effect of $CaCl_2$ concentration on bead intensity and neo-FOS production (Fig. 3): Bead intensity increased as $CaCl_2$ concentration increased, but the maximum neo-FOS production was obtained at 0.2 M $CaCl_2$. This result implie that a high $CaCl_2$ concentration could cause the formation of too strong an alginate gel structure, which prevents the diffusion of substrate, thus inhibiting the reaction with immobilized cells on alginate beads.

Effect of Glucose Oxidase on Neo-FOS Production

A number of studies have reported that glucose, a byproduct of FOS production, inhibits the transfructosylating reaction

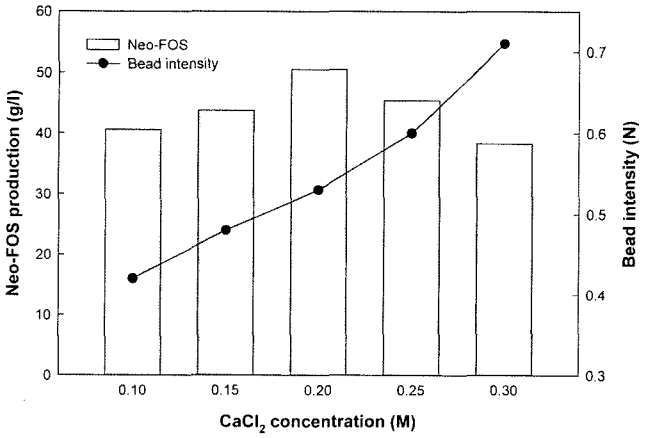

Fig. 3. Effect of $CaCl_2$ concentration on bead intensity and neo-FOS production.

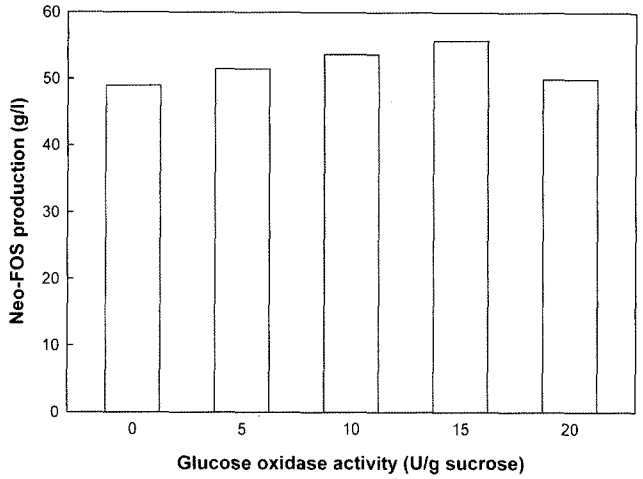

Fig. 4. Effect of amount of glucose oxidase on neo-FOS production in the batch reaction.
A free enzyme solution of glucose oxidase was added to 100 ml of sucrose solution (60%, w/v) containing 10 g of cell-immobilized beads, and allowed to produce neo-FOS at 50°C for 24 h at 100 rpm in a shaking incubator.

[4, 17, 19]. Reaction rates increase when glucose is removed, and high FOS yields are obtained. To improve FOS productivity, a mixed enzyme system has been employed using a commercial enzyme, either glucose oxidase or catalase. In the present study, the effect of glucose oxidase was investigated in batch production. To determine the optimal amount of glucose oxidase, a batch reaction using cell immobilized beads and various amounts of glucose oxidase was used. As shown in Fig. 4, maximum neo-FOS production (55.8 g/l) was obtained when 15 units of glucose

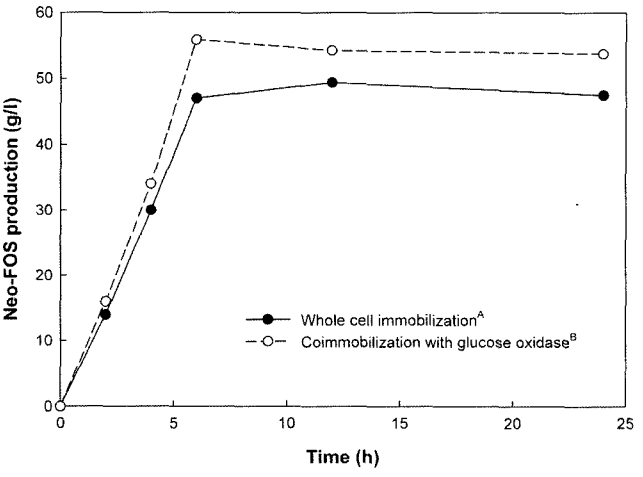

Fig. 5. Comparison of neo-FOS production for whole cell immobilization and co-immobilization with glucose oxidase.
[A]Mycelia of *P. citrinum* were immobilized in calcium alginate. [B]Mixture of mycelia and 15 units of glucose oxidase activity per gram of sucrose was immobilized in calcium alginate. Ten grams of immobilized beads were allowed to produce neo-FOS at 50°C for 24 h at 100 rpm in a shaking incubator.

oxidase was added. Sheu *et al.* [19] also suggested that 15 units of glucose oxidase activity per gram of sucrose might be optimal for the production of FOS in large-scale production. Therefore, to produce a high yield of neo-FOS, a mixture of cells and glucose oxidase was entrapped in alginate beads, and neo-FOS production was then compared with the whole-cell immobilization process. In batch production, co-immobilization had a higher productivity (9.317 $gl^{-1}h^{-1}$) than whole-cell immobilization (4.167 $gl^{-1}h^{-1}$) (Fig. 5). Thus, it appears highly likely that neo-FOS can be produced in high yields by large-scale production based on a mixed immobilization process.

Acknowledgments

This study was supported by research grants from the Korea Science and Engineering Foundation (KOSEF) through the Applied Rheology Center (ARC), an official KOSEF-created Engineering Research Center (ERC) at Korea University, Seoul, Korea.

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