Effect of Sucrose Concentration and Cultivation Time on Batch Production of Fructosyltransferase by *Aureobasidium pullulans* CCY 27-1-1194*

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The effect of the initial sucrose concentration (50 g dm^{-3}, 200 g dm^{-3}, and 350 g dm^{-3}) and time of cultivation (1 d, 2 d, and 4 d) on the fructosyltransferase production by *Aureobasidium pullulans* CCY 27-1-1194 in shaken flask cultivations was investigated. It was found that the overall fructosyltransferase production depended strongly on the initial sucrose concentration when the highest value of enzyme activity (130 000 U dm^{-3}) was achieved at 350 g dm^{-3} in the fourth cultivation day. The maximum of specific activity per dry cell mass was achieved at the same sucrose concentration, but already in the second day. Since the second cultivation day, the ratio of the enzyme activity contained in cells and released into medium was 1.3—1.6, independently of sucrose concentration. In order to discuss the fructosyltransferase production in relation to cell growth and state of the culture, the sucrose conversion, produced amount of fructooligosaccharides and organic acids, and the yield factor of biomass on substrate, were determined as well.

The role of fructooligosaccharides (FOS's) in human nutrition is admittedly beneficial for health and these oligosaccharides are classified as prebiotics [1, 2]. Many food companies over the world use fructooligosaccharides as food ingredients in various food products. Industrial production of short-chain FOS's is based on transformation of sucrose by enzymes with a transfructosylating activity – fructosyltransferases (FTases). Microorganisms such as *Aspergillus niger*, *Aspergillus japonicus,* and *Aureobasidium pullulans* are the most important industrial sources of high-activity fructosyltransferases [3, 4].

Aureobasidium pullulans (*AP*) is an ubiquitous saprophyte classified under ascomycetes, family Dothideales [5]. Among other important enzymes, this organism produces fructosyltransferase of 1^F-type, which catalyzes the transformation of sucrose to short-chain fructooligosaccharides with $\beta(1\rightarrow2)$-glycosidic bonds between fructosyl units. FTase is produced by aerobic cultivation of the microorganism at appropriate growth conditions in both intracellular and extracellular forms. Investigations of the effect of the cultivation media composition and the time of cultivation on the FTase production by strains of *Aureobasidium pullulans* were performed by *Hayashi et al.* (*AP* ATCC 20524) [6] and *Jung et al.* (*AP* KFCC 10245) [7]. They concluded that sucrose was the best carbon source promoting the FTase production in the concentration range of 100—250 g dm^{-3}, although the maximal cell growth was achieved at 50—100 g dm^{-3} of the initial sucrose concentration. Yeast extract at 15 to 20 g dm^{-3} was found the best nitrogen source [6]. Addition of $NaNO_3$ (10—20 g dm^{-3}), $MgSO_4 \cdot 7H_2O$ (0.5—2 g dm^{-3}), and K_2HPO_4 (5—7.5 g dm^{-3}) to the cultivation medium resulted in the increase of the production of intracellular enzyme. The optimal cultivation temperature was between 28 °C and 30 °C in all experiments. After 48 h of cultivation, the cell growth reached the stationary phase and the overall FTase production was stopped [6].

In the recent period, we have been concerned with the investigation of a 1^F-fructosyltransferase (1^F-FTase), an enzyme produced in intracellular and extracellular forms during aerobic cultivation of *Aureobasidium pullulans* CCY 27-1-1194. This enzyme has been shown to have a high activity, stability, and regioselectivity for the production of 1^F-type FOS's [8, 9]. In this study, we decided to optimize the FTase production since previous studies of other authors demonstrated that optimal medium compositions somewhat

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differed for the two strains of *Aureobasidium pullulans* investigated [6, 7]. Two parameters, the initial sucrose concentration, and the cultivation time, respectively, were examined for their effect on the FTase production in shaken flask cultivations.

EXPERIMENTAL

The analytical standards of 1-kestose, nystose, and 1^F-fructofuranosylnystose were obtained from Wako Pure Chemical Industries (Osaka, Japan); other chemicals used as standards were purchased from Fluka (Buchs, Switzerland) and Sigma (Deisenhofen, Germany). All other chemicals were of anal. grade and were obtained from readily available commercial sources.

Aureobasidium pullulans CCY 27-1-1194 (Culture Collection of Yeasts, Bratislava, Slovakia) was maintained on 15 g dm^{-3} agar slants containing 10 g dm^{-3} of sucrose and 53 g dm^{-3} of malt extract. The inoculum was grown in 50 cm^3 of a seed medium consisting of 2 g dm^{-3} of yeast extract and 10 g dm^{-3} of sucrose with pH 5.2 in a 100 cm^3 flask shaken at 28 °C for 48 h.

The analyses of saccharides were performed by HPLC (Knauer, Germany) as it was previously described [9]. In the ion-exclusion mode, the column Watrex Polymer IEC Pb form, 250 mm × 8 mm i.d. (Watrex Prague, Czech Republic) was maintained at 80 °C and double distilled water was used as the mobile phase at a flow rate of 0.8 cm^3 min^{-1}. The column Separon SGX C18, 150 mm × 3 mm i.d. (Tessek Prague, Czech Republic) and double distilled water (1 cm^3 min^{-1}) were used in the reversed-phase mode. The amount injected was 10 mm^3 and the detection was performed by a differential flow refractometer at 25 °C.

A base-line separation of the saccharides contained in the cultivation media was not possible on either of the above HPLC columns. The ion-exclusion column separated sucrose, glucose, and fructose very well but the resolution of fructooligosaccharides was poor. On the contrary, the reversed-phase column had an excellent separation efficiency for FOS's but did not separate monosaccharides at all. Combining these two modes of HPLC, a complete spectrum of saccharides was determined.

Organic acid content was analyzed by HPLC using a Watrex Polymer IEC H form column, 250 mm × 8 mm i.d. (Watrex Prague, Czech Republic) at 50 °C with 9×10^{-3} M-H_2SO_4 as an eluent at a flow rate of 0.8 cm^3 min^{-1}. The peaks were detected by an UV spectrophotometer at $\lambda = 214$ nm.

Cultivation

The cultivation medium contained (ρ/(g dm^{-3})): yeast extract – 10, $NaNO_3$ – 10, $MgSO_4$ – 0.5, K_2HPO_4 – 5, chloramphenicol – 0.1, and sucrose – 50, 200 or 350. The pH was adjusted to 6.5 before sterilization. 1 cm^3 of inoculum was transferred into 100 cm^3 of cultivation medium in a 500 cm^3 flask and the flasks were placed on a rotary shaker and agitated at the frequency of 180 min^{-1} and temperature of 28 °C for 24—96 h. At the end of cultivation, a 10 cm^3 sample was taken off from the cultivation broth, filtered through a 0.2 μm membrane filter, washed with deionized water and dried to a constant mass to obtain dry cell mass. The filtrate was used for the analysis of saccharide content by HPLC. The cells were harvested by filtration of the rest of the cultivation broth through a sintered glass filter with 5—15 μm pores, and washed with a physiological solution. The filtrate was used for activity assay in the medium without any further treatment.

Activity Assay

The enzyme activity was assayed by the modified method published previously [9]. The reaction mixture consisted of 9.5 cm^3 of 736.8 g dm^{-3} sucrose in 0.1 M-citric acid/K_2HPO_4 buffer (pH 5.5) and 0.5 cm^3 of an enzyme preparation (final sucrose concentration was 700 g dm^{-3}). The enzyme preparation was either a filtrate of culture broth for the cultivation medium activity assay or wet cells appropriately diluted in the citric acid/K_2HPO_4 buffer for the cell activity assay. The assay was carried out in a stirred batch reactor at 55 °C. Samples (1 cm^3) of the reaction mixture were taken off in predefined time intervals and the reaction was stopped by boiling in a water bath for 2 min. The samples were then diluted and filtered through a membrane filter and frozen before analysis. One unit of transfructosylation activity was defined as the amount of enzyme which catalyzes the formation of 1 μmol of kestose in 1 min under these conditions.

RESULTS AND DISCUSSION

The effect of sucrose concentration and time of cultivation on cell growth is illustrated in Fig. 1. The cell growth was strongly inhibited by sucrose concentration when the growth was the fastest during the first two days at the initial sucrose concentration, ρ_{S0}, 50 g dm^{-3}. The growth then continued in all cases up to the fourth day of cultivation but at the lowest initial sucrose concentration, it was probably limited by the low residual sugar concentration. However, the amount of biomass obtained after four days of cultivation was almost the same, 14—16 g dm^{-3} of dry cells, at all initial sucrose concentrations. This amount was similar to that reported by *Yun et al.* [10] for *AP* KFCC 10524, but lower than 22—25 g dm^{-3} obtained for *AP* ATCC 20524 [6] and *AP* KFCC 10245 [7] in cultivations with 150—200 g dm^{-3} of sucrose. *Jung et al.* [7] found that the maximum cell concentration

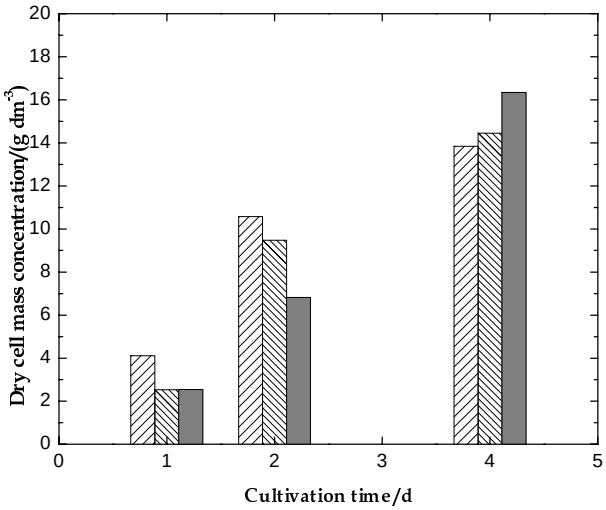

Fig. 1. Effect of time of cultivation and initial sucrose concentration (▨ 50 g dm^{-3}, ▩ 200 g dm^{-3}, ■ 350 g dm^{-3}) on cell growth.

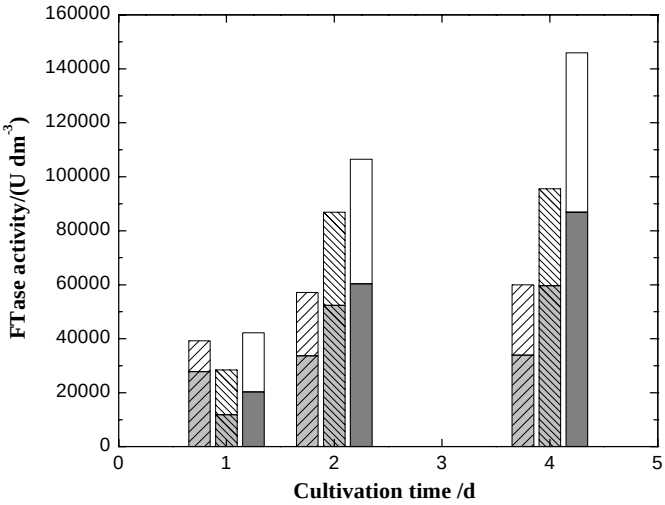

Fig. 2. Effect of time of cultivation and initial sucrose concentration on the FTase production. The bottom parts of bar diagram represent the fraction of FTase activity of cells (ρ_{S0} = ▨ 50 g dm^{-3}, ▩ 200 g dm^{-3}, ■ 350 g dm^{-3}) and the top parts the fraction of activity released into the medium (ρ_{S0} = ▨ 50 g dm^{-3}, ▩ 200 g dm^{-3}, □ 350 g dm^{-3}).

was reached with sucrose concentration of 100 g dm^{-3} and higher sucrose concentration decreased the final cell mass, but our results did not show this tendency. It is worth to mention that the cell growth probably did not reach the stationary phase in the cultivations with 200 g dm^{-3} and 350 g dm^{-3} of sucrose. Since the residual amount of carbohydrates in the cultivation medium was nonzero, it might be possible to obtain somewhat higher amounts of biomass in a prolonged cultivation time.

Fructosyltransferase Production

The overall production of FTase depended strongly on the initial sucrose concentration (Fig. 2). This effect was the most notable at ρ_{S0} = 50 g dm^{-3} where the production of FTase was stopped after two days. At ρ_{S0} = 200 g dm^{-3}, the relative increment of the total FTase activity between the 2nd and 4th day was much lower in comparison with that between the 1st and 2nd day. Such a drop of the enzyme production rate was not observed in the cultivations with the initial sucrose concentration of 350 g dm^{-3} where the total enzyme activity reached the value of 131 000 U dm^{-3} in the fourth day. No suppression of FTase production by increasing sucrose concentration was observed, which is contrary to the results of *Hayashi et al.* who found the largest amount of enzyme produced at 100—200 g dm^{-3} of sucrose after two cultivation days [6].

The FTase activity of cells represented approximately 60—70 % of the total activity since the second cultivation day and the ratio of activities of cells and activities in cultivation medium was 1.3—1.6 independently of the sucrose concentration. This observation differs from those obtained by *Hayashi et al.* [6, 11] but it is similar to that of *Jung et al.* [7] for different strains of *Aureobasidium pullulans*. The ratio of the cell to cultivation medium activities depends on the content of magnesium sulfate in the production medium. *Jung et al.* reported that the addition of magnesium sulfate to the medium at the content of 0.2 % increased this ratio to the value of about 1.2 which was almost constant during the entire cultivation period [7]. From this point of view, the value of the ratio of 1.3—1.6 obtained by us at 0.05 % $MgSO_4$ is noteworthy.

The specific cell activity with respect to dry cell mass is a crucial factor for the control of a cultivation run if whole cells, either free or immobilized, are used as biocatalysts. Its value reached the maximum already in the first day at ρ_{S0} = 50 g dm^{-3} or in the second day at ρ_{S0} = 200 g dm^{-3} and 350 g dm^{-3} as shown in Fig. 3. The maximum value of 8860 U g^{-1} was reached again in the cultivation with initial sucrose concentration of 350 g dm^{-3}.

As it has been mentioned above, the initial sucrose concentration influenced the amount of produced FTase whereas the cell mass produced after four cultivation days was unaffected. This result suggests that the FTase production was promoted by high sucrose concentrations. Although other authors used different activity assay conditions and the absolute values are not fully comparable, the FTase activities of *AP* CCY 27-1-1194 are of the same order of magnitude as those published for highly active production strains, which suggests a potential of our strain for industrial production of fructosyltransferase.

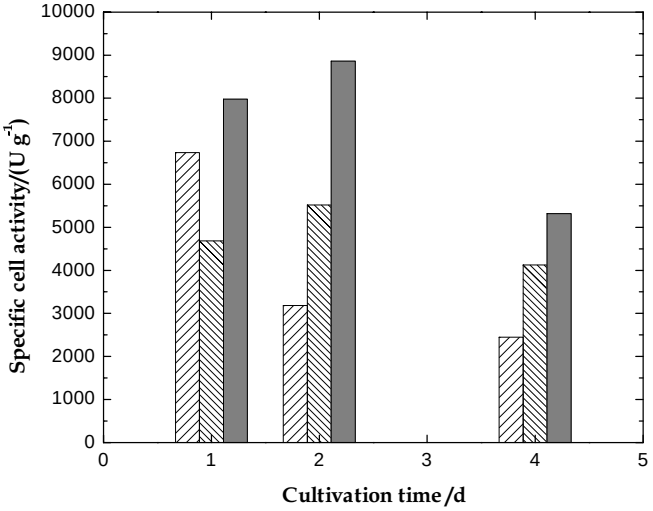

Fig. 3. Effect of time of cultivation and initial sucrose concentration on the specific cell FTase activity per mass of dry cells. The symbols are the same as in Fig. 1.

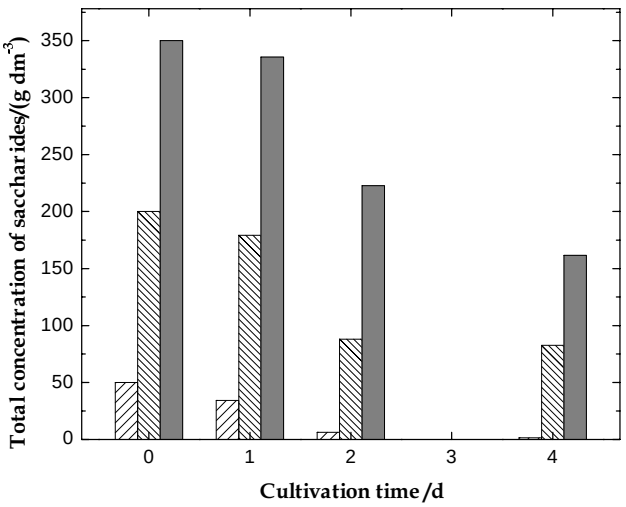

Fig. 4. Effect of time of cultivation and initial sucrose concentration on the total concentration of saccharides in the cultivation medium. The symbols are the same as in Fig. 1.

Sucrose Transformation

It follows from the analyses of saccharides in the cultivation medium that most sucrose was rapidly transformed within the first day and, at the same time, D-glucose and FOS's concentrations were maximal. The changes in the content of sucrose, D-glucose, D-fructose, and individual fructooligosaccharides are shown in Table 1. In the cultivations with 200 g dm^{-3} and 350 g dm^{-3} of sucrose, about 52—56 % of FOS was accumulated during the first day in the cultivation medium with nystose and 1^F-fructofuranosylnystose as predominant oligosaccharides. Surprisingly, the decrease of 1^F-fructofuranosylnystose concentration was much slower than that of shorter-chain FOS's as it is visible at ρ_{S0} = 200 g dm^{-3} and 350 g dm^{-3}. From monosaccharides, D-glucose was utilized more rapidly than D-fructose and the residual concentrations of monosaccharides increased with higher initial sucrose concentrations. Fig. 4 shows the total residual amounts of saccharides in the medium during the cultivations. The spectrum of saccharides in the cultivation medium and the changes of its composition during the cultivation run were similar to the results reported by *Yun* [4] and *Hayashi et al.* [11].

Since sucrose is not the only carbon source during the cultivation, as it is transformed into mono- and oligosaccharides, the sum of all saccharides in the medium was considered as the substrate. Then the conversion of substrate by the cells was calculated from the relationship

$$X_S = \left[V_0 c_{S0} - V_\infty \left(\frac{c_{G\infty} + c_{F\infty}}{2} + c_{S\infty} + \right.\right.$$
$$\left.\left. + \frac{3c_{GF_2\infty}}{2} + 2c_{GF_3\infty} + \frac{5c_{GF_4\infty}}{2} \right)\right] \Big/ V_0 c_{S0} \quad (1)$$

Table 1. Saccharide Content in the Cultivation Medium during the Cultivation Determined by Ion-Exclusion and Reversed-Phase HPLC

ρ_{S0}	Time	ρ_S	ρ_G	ρ_F	ρ_{GF_2}	ρ_{GF_3}	ρ_{GF_4}	FOS's mass fraction*
g dm^{-3}	d	g dm^{-3}	g dm^{-3}	g dm^{-3}	g dm^{-3}	g dm^{-3}	g dm^{-3}	%
50	1	2.32	11.04	11.56	1.60	4.56	3.32	27.6
	2	0.64	0.32	5.40	0	0	0	0
	4	0.24	0.76	0.60	0	0	0	0
200	1	9.52	60.36	15.48	16.08	47.72	30.16	52.4
	2	1.56	29.20	46.60	1.96	3.24	5.56	12.2
	4	0.67	2.01	78.95	0.56	0.17	0.23	1.2
350	1	41.76	111	14.96	40.56	104.48	42.92	56.0
	2	7.48	95.72	47.40	7.19	15.92	49.04	32.4
	4	2.76	55.84	90.44	3.61	2.40	6.56	7.8

*The FOS's mass fraction is related to the total content of saccharides in the cultivation medium. Abbreviations: G – D-glucose, F – D-fructose, S – sucrose, GF_2 – 1-kestose, GF_3 – nystose, GF_4 – 1^F-fructofuranosylnystose.

where V_0 and V_∞ are the volumes of the cultivation medium at the start and at the end of cultivation (in dm^3) and c_{S0} is the initial sucrose concentration (in mol dm^{-3}), $c_{i\infty}$ are the final concentrations of saccharides (in mol dm^{-3}) where the index i represents the following compounds: G – D-glucose, F – D-fructose, S – sucrose, GF_2 – 1-kestose, GF_3 – nystose, and GF_4 – 1^F-fructofuranosylnystose. Almost all saccharides (substrate conversion of 97 %) were utilized after two days in the cultivation at ρ_{S0} = 50 g dm^{-3}. In the cultivations with 200 g dm^{-3} and 350 g dm^{-3} of sucrose, the substrate conversions at the end of the cultivation period were 70 % and 62 %, respectively.

The yield factor of biomass on substrate, Y_{XS}, was calculated as the ratio of the dry cell mass to the mass of total saccharides consumed (nominator of eqn (*1*)). Fig. 5 shows that Y_{XS} values were essentially independent of the cultivation time but differed significantly with regard to ρ_{S0} where the maximum value of about 0.27 was reached at the cultivations with ρ_{S0} = 50 g dm^{-3}. Y_{XS} values were considerably lower at higher initial sucrose concentrations. This indicates a shift in the metabolism in comparison with cultivations at the low sugar concentrations where respiratory pathways are more efficiently employed.

In addition to the transformation of sucrose into other saccharides and its utilization for cell growth and protein production, some organic acids were formed during the cultivation of *Aureobasidium pullulans*. The resultant pH's of cultivation media after four-day cultivations were approximately 5.5—6.0. We also determined the spectrum and total content of organic acids in the cultivation medium at the end of cultivations. A significant formation of organic acids was found in all cases. The main organic acids identified by HPLC analyses were pyruvic, D-gluconic, succinic, and fumaric. The overall organic acids content increased with the cultivation time and initial sucrose concentration (Fig. 6). This result indicated an obvious correlation between the total saccharide consumption and organic acid production.

The production of organic acids was not directly coupled with the cell growth. It is known that pH decreases during the cultivation and it is important to keep it above 5.5 for optimal cell growth [4]. The organic acids production and related pH decrease have not been investigated in detail so far. In our case, even the highest amount of organic acids produced at ρ_{S0} = 350 g dm^{-3} had no apparent negative effect on the cell mass production while the pH remained above 5.5. Further, more thorough studies should, however, be made to account for the effects of organic acids and pH.

The results of our study of the effect of initial sucrose concentration and cultivation time on fructosyltransferase production by *Aureobasidium pullulans* CCY 27-1-1194 showed that significantly larger sucrose concentrations than previously predicted are

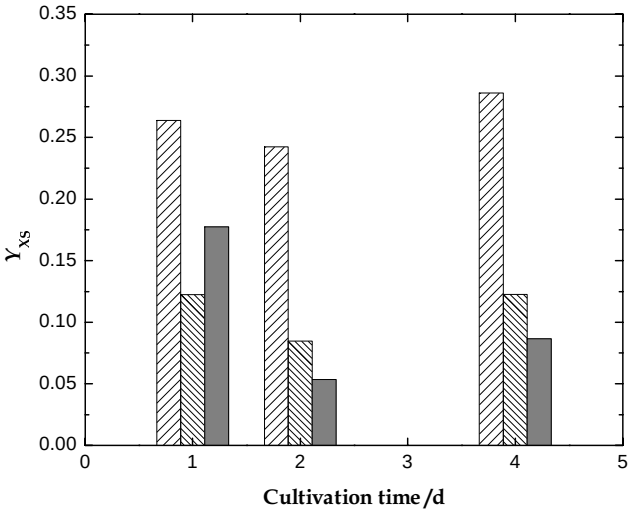

Fig. 5. Effect of time of cultivation and initial sucrose concentration on the yield factor of biomass on substrate. The symbols are the same as in Fig. 1.

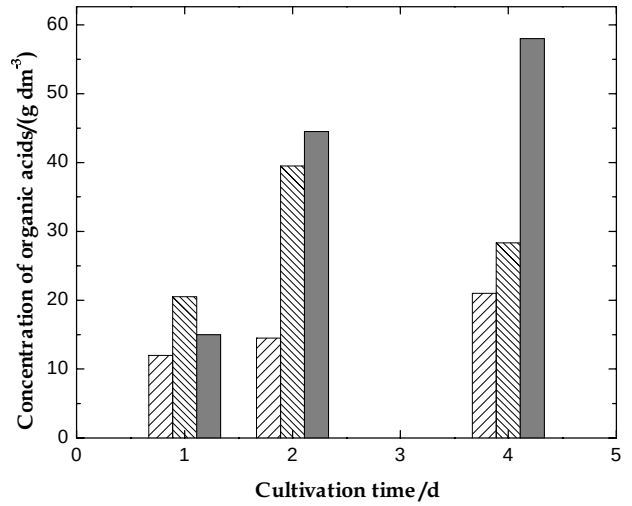

Fig. 6. Effect of time of cultivation and initial sucrose concentration on the production of organic acids. The symbols are the same as in Fig. 1.

better for the maximization of both the total amount of enzyme produced and the specific FTase activity of whole cells. Furthermore, it was found better not to proceed the cultivation to high degrees of sucrose conversion when the maximization of cell specific activity is of importance. Two days of cultivation seemed to be a good period for obtaining relatively high amount of cells with maximal activity.

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