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Full Length Research Paper

Production and application of glucose-fructose oxidoreductase for conversion of pineapple juice sugars

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This work is concerned with the production of glucose-fructose oxidoreductase (GFOR) enzyme for converting pineapple juice sugars into dietetic derivatives. GFOR was produced from *Zymomonas mobilis* by sub-merge fermentation of 10 L. During fermentation, the highest biomass formation was observed at 24 h and the highest enzyme activity per g biomass was observed at 27 h of fermentation. Cells were isolated by centrifugation, broken down by high pressure homogenizer, enzyme separated by centrifugation and finally the enzyme was purified on a dye substituted column. Electrophoresis of separated fractions confirmed its structure showing a band at 40 kD. Application of GFOR successfully converted pineapple juice sugars; fructose and glucose into sorbitol, a low calorie sugar alcohol, and gluconic acid, respectively. This enzyme was found highly efficient in case of converting juice sugars of fruits juices having higher natural pH.

Key words: Glucose-fructose oxidoreductase, fermentation, conversion, pineapple juice sugars.

INTRODUCTION

Next to moisture, carbohydrates are the major and important components in almost all fruit juices (http://winemaking.jackkeller.net/sugar.asp). Sucrose, glucose and fructose are the most abundant and predo-minant carbohydrates in fruit juices. In case of pineapple juice, the most important soluble carbohydrates are sucrose, fructose and glucose (glucose/fructose 0.8 - 1.1% and sucrose 42%). From nutritional point of view, fruit juice sugars are of importance as source of dietary energy. They also give taste to beverages. Apart from these, juice sugars may have negative affect on human health by contributing to weight gain, decreasing obesity and dental caries (Baer, 1989; Matsukubo and Takazoa, 2006; Schulze et al., 2004; Wideman et al., 2005). For consumers who wish to control their weight or other problems related to high sugar consumption, researchers have been developing alternative products with low or even without sugars (Acosta et al., 2006), and also sucrose substitutes like isomaltulose (Cho et al., 2007) or tagatose (Haltrich et al., 1998). Low sugar carbonated beverages produced with high sweeteners although available in market, are the subject of much debate (Beverage World, 2004). Furthermore, up to date there is no replacement for fresh fruit juice taste and other beneficiary components like antioxidants present in juices (Oszmianski et al., 2007). All the sugar alcohols like sorbitol, mannitol, lactitol, xylitol etc contain lower calories and they are found incompletely absorbed into the blood stream by the small intestine resulting in a smaller change in blood sugars. Moreover, these sugar alcohols are not metabolized by oral bacteria, so they do not con-tribute in dental caries either. They are almost as sweet

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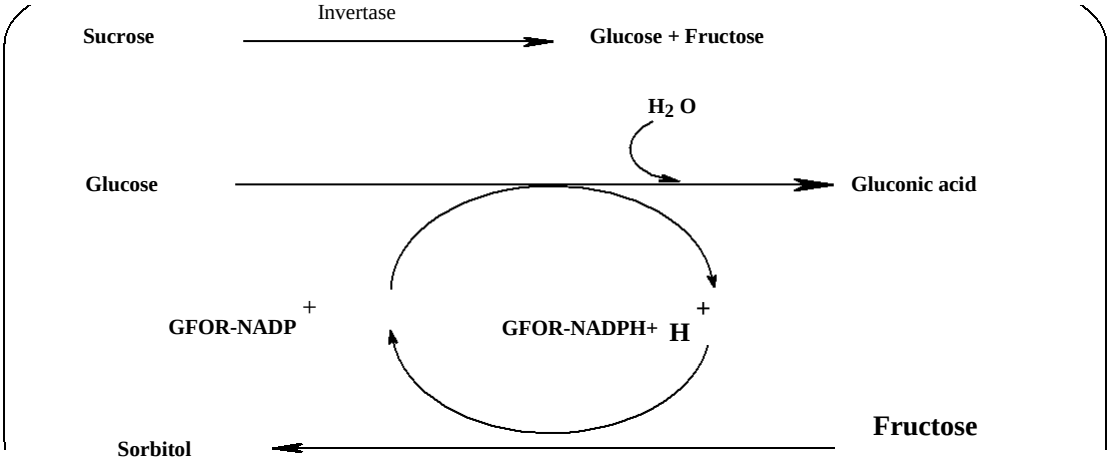


Figure 1. Schematic diagram of enzymatic conversion of juice sugars by GFOR (Aziz et al., 2011).

as sugar but they contain about 40% less calories than the sugar they originated from. Therefore, complete conversion of juice sugars into sugar alcohols will contribute to roughly 40% calorie reduction, whereas calorie reduction should be 25% of its original as to say low calorie products (Ashurst, 1998). Therefore, the production of dietetic fruit juices by keeping other compounds unaffected is only possible by biotechnology like by using cell free enzymes (Aziz et al., 2011). In this case, glucose-fructose oxidoreductase might be an excellent choice for converting natural juice sugars into dietetic derivatives.

GFOR (EC 1.1.99.28), a periplasmatic (Loos et al., 1996) enzyme responsible for sorbitol formation by *Zymomonas mobilis*, has been first identified, isolated and characterized by Zachariou and Scopes (1986). The enzyme operates by a ping-pong mechanism, catalyzing the reaction of one of its substratrs to yield a product that dissociates before the other substrates binds. Hence the overall reaction consists of two half reactions, with alternate reduction of the bound $NADP^+$ (as glucose is oxidized to gluconolactone) and oxidation of NADP (as fructose is reduced to sorbitol) (Figure 1). The gluconolactone is subsequently converted to glucorunic acid; however, sorbitol is not further metabolized by the cell. The biotechnological potential of GFOR has been recognized and considerable efforts have been undertaken to optimize the enzymatic production of sorbitol and gluconic acid. One of the great advantage of this process ascribed that there is no need to add the expensive and instable coenzyme NAD (P)H, its recycling and retention in the reaction system during continuous operation (Howaldt et al., 1990; Ikemi and Ishimatsu, 1990). Another advantage it is necessary minimum enzyme purification steps due to this bind coenzyme.

The continuous production of sorbitol and gluconic acid by using permeabilized cells of *Z. mobilis* was described in many reports (Chun and Rogers, 1988; Rehr et al., 1991). The application of cell free GFOR was found confined to the continuous production of sorbitol and gluconic acid by using sucrose or glucose and fructose as substrates (Nidetzky et al., 1996; Fürlinger et al., 1998; Gollhofer et al., 1995). Enzymes are usually specific and a single enzyme usually converts one substrate to product. As juices contains one more sugars, it is necessary a blend of enzymes to convert all the sugars which is not economical in practical point of view. Oxidoreductase (like glucose-fructose oxidoreductase) which converts more than one compound to its derivatives seems to be feasible for dietetic juice production. Therefore, the objectives of the study was to produce , purify and characterize of cell free glucose fructose oxidoreductase (GFOR) enzyme and its application for the batch convert ion of pineapple juice sugars into dietetic derivatives.

MATERIALS AND METHODS

Fermentation of *Z. mobilis*

Z. mobilis (DSM 473), used for fermentation, was maintained in liquid medium at 4 °C. The bacteria was cultivated anaerobically in an automatically controlled 30 L fermenter fitted with a pH electrode for 27 h at 30°C, 130 rpm and at pH 6.2 (Applikon®, The Netherlands). pH was adjusted with 5M KOH. The Medium used for maintenance, inocula production and fermentation experiments contained 200 g/L glucose, 5 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.02 g/L $MgSO_4x7H_2O$, 0.02 g/L $Fe(NH_4)_2(SO_4)_2x6H_2O$, 0.001 g/L biotin and 0.002 g/L calcium panthotenate.Glucose was autoclaved separately. Yeast extract, KH_2PO_4 and magnesium sulphate were autoclaved together. The last three components, ammonium ferrous sulphate and the vitamins were added by sterile filtration. Growth media were inoculated with 1 L pre-culture grown on the same medium.

Cell separation and enzyme extraction

Cells were diluted 1:2 to 1:3 (by weight) in buffer (10 mM K-mes, pH 6.3) and disintegrated by using high pressure homogenizer at 1000 bar. Cells were cooled at 4°C and the cooling chamber of the

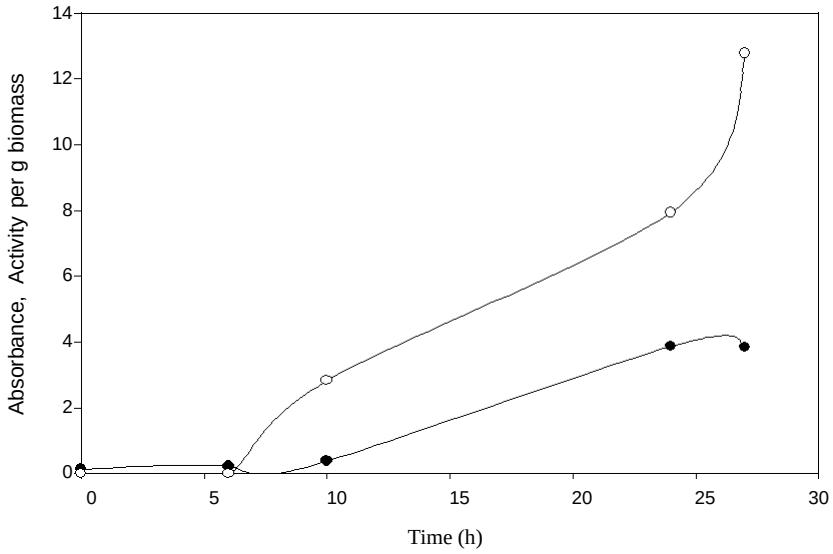


Figure 2. Growth of *Zymomonas mobilis* in standard medium, temperature 30, 110 rpm, pH 6.2. Solid circle represents absorbance at 660 nm and blank circle represents activity of GFOR/g biomass. pH was controlled by addition sterile 2M KOH.

homogenizer was turn on before 30 min as the temperature of the homogenizer increased drastically during homogenization. To achieve a proper extraction of the cells, the cell suspension was passed 3 times. In order to avoid heat denaturation, the disintegrate suspension was collected on ice. The extraction buffer, the suspended cells and the extracted cells were cooled in the same manner. After extraction the resulting cell debris were removed by ultracentrifugation (Ti 45, 30000 rpm, 4°C, 45 min). The rotor and the centrifugal chamber were cooled down to 4°C to avoid heat denaturation. The resulting supernatant was termed crude extract and protein content and enzyme activity of it was determined.

Enzyme purification

The crude cell extracts were purified on a Sepharose CL-4B column with adsorbed yellow MX-GR (ICI reactive yellow) as described by Fürlinger et al. (1998). Fractions with GFOR activity were concentrated by diafiltration on Amicon 10 kDa cell. For SDS-PAGE electrophoresis, the samples and standard protein were diluted with sample buffer (Laemmli) and boiled for 5 min. Using the Phast Gel sample applicator 8/1 a total sample 4 µL was applied to the purchasable Phast Gel Gradient 8-25. The gradient gel contained 8-25 g acrylamide and 2 g bisacrylamide per 100 mL gel. The SDS buffer strips consisted of 2-8% agarose and contained 0.88 M L-Alanin and 0.25 M Tris, pH 8.8. Precision plus protein standard (dual colour) was used as protein standard.

Batch conversion

Simultaneous conversion of mixtures of glucose and fructose of pineapple juice were carried out at 25°C using 1.2 U GFOR/mL at a constant pH (maintained by the controlled addition of 1 M potassium hydroxide; Gamma/4-I; Prominent, Heidelberg, Germany). The batch arrangement consisted of magnetic stirrer under controlled temperature and pH. At specific time interval 0.5 mL sample was taken, heated for 10 min at 99°C, centrifuged at 13,000 rpm and then analyzed by HPLC.

Enzyme activity

The activities of GFOR was quantified at 25°C as described by Zachariou and Scopes (1986) and Gollhofer et al. (1995) by following the pH dependent decolorization of 0.3 mM p-nitrophenol solution at 405 nm due to the formation of gluconic acid. One unit of enzyme activity refers to 1 µmol gluconic acid formed/min by the action of either GFOR/gluconolactonase or gluconolactonase only. All rates are corrected for non-enzymatic hydrolysis of D-gluconolactone in the absence of lactonase at 25°C. The extinction coefficient was calculated from the extinction change caused by 1 µM acid under standard conditions. An extinction coefficient of 0.211 $mM^{-1}cm^{-1}L^{-1}$ was calculated. Protein determinations were done according to the dye binding method of Bradford (1976) using BSA as standard.

Sugar analysis

Substrates and products in the reaction mixture were determined by HPLC using an Aminex HPX-87C column, operated at 85°C. The eluent was 10 mM calcium nitrate at the flow rate of $0.7mLmin^{-1}$ and refractive index (RI) detection was used. The dilution of the reaction mixture because of alkali addition during turnover was accounted for by means of the mass balance of substrates and products.

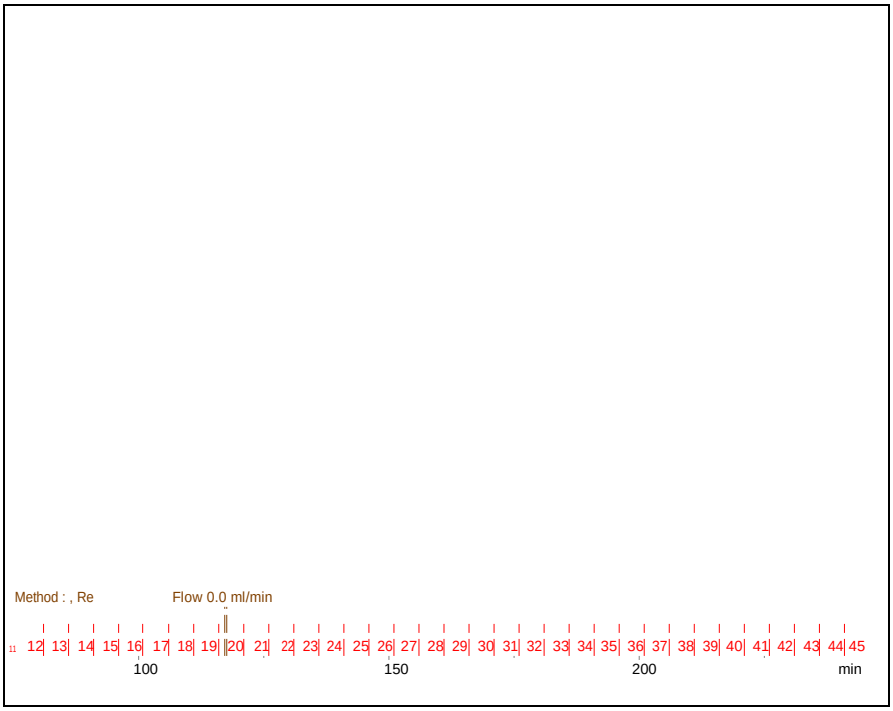
RESULTS AND DISCUSSION

Cell growth

During fermentation *Z. mobilis* cell growth was initiated after 10 h of fermentation and no further biomass formation was observed after 24 h fermentation (Figure 2) . Although highest biomass formation was observed at 24 h the highest enzyme activity per g biomass was observed at 27 h of fermentation. Fürlinger et al. (1998)

Table 1. Purification of GFOR from *Zymomonas mobilis.*

Purification steps	Total activity (U)	Protein load (mg)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Raw extract	1368	-	2325	0.6	100
Dye column	1144	110	92.43	12.38	84
Source S	52	20.54	0.72	72	20



Elution volume (mL)

Figure 3. Elution chart of GFOR applied to Sepharose CL-4B column with adsorbed yellow MX-GR.

found the highest bio-mass production was found at 20 h of fermentation. In time-dependent growth of *Z. mobilis* a lag phase of up to 6 h was observed. Duration of the exponential phase was about 14 h and the stationary phase was reached at 24 h. It is interesting that pre-cultures prepared from aged stock culture surprisingly did not show any GFOR activity. Biomass formation in the media fermented with aged pre-culture and fresh pre-culture was observed the almost same but no activity was found in the biomass produced by aged pre-culture.

Enzyme prepration

The volumetric activity and specific activity of GFOR in crude extract was very low as shown in Table 1. The two step purification procedure yielded homogenous protein. Although pure GFOR had a specific activity of 72 U/mg the yield of enzyme was very low (20%). For industrial application additional purification steps would result in higher cost. In previous studies it showed that purification on dye yellow column was sufficient for conversion studies (Gollhofer et al., 1995). Purification on dye column yielded 84% enzyme, and its specific activity is 20 times higher than the crude enzyme. The chromatogram of GFOR purification on dye column is shown in Figures 3.

According to the chromatogram, the highest GFOR activity was found in fractions 28, 29 and 30. The electrophoresis of these fractions revealed one band as shown in Fig. 4. It is reported that GFOR from Z. *mobilis* DSM 473 is a tetramer at pH 5.0 (Zachariou and Scope, 1986; Loos et al., 1996). At pH 7.0 an octameric association was also reported (Zachariou and Scopes, 1986).

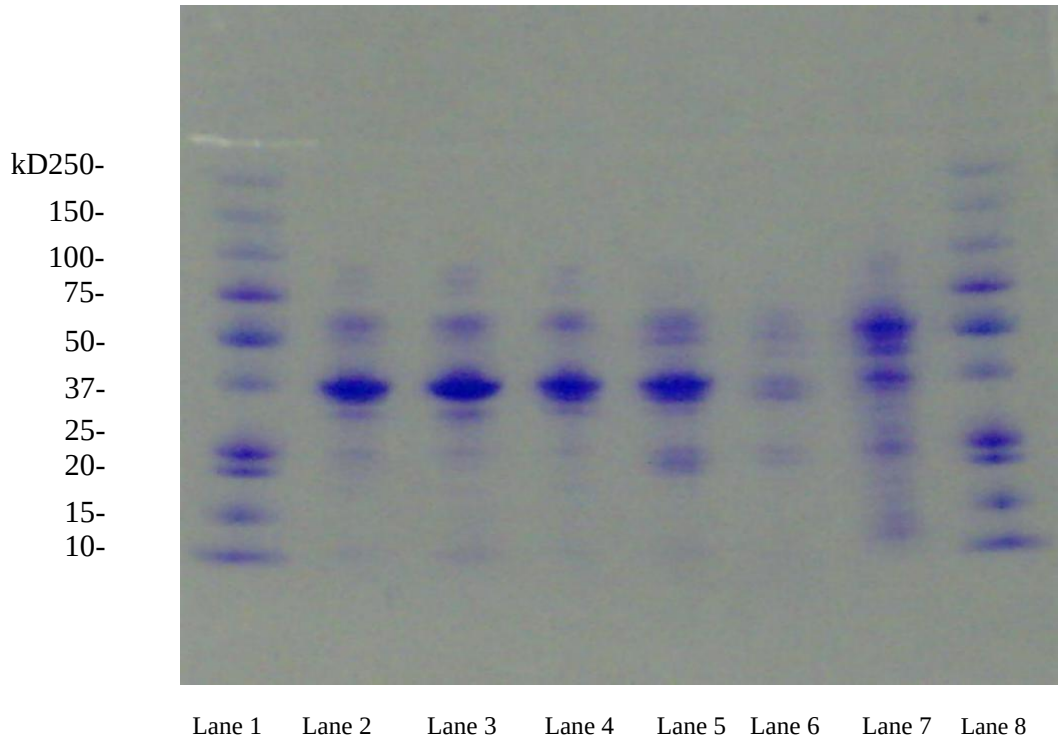


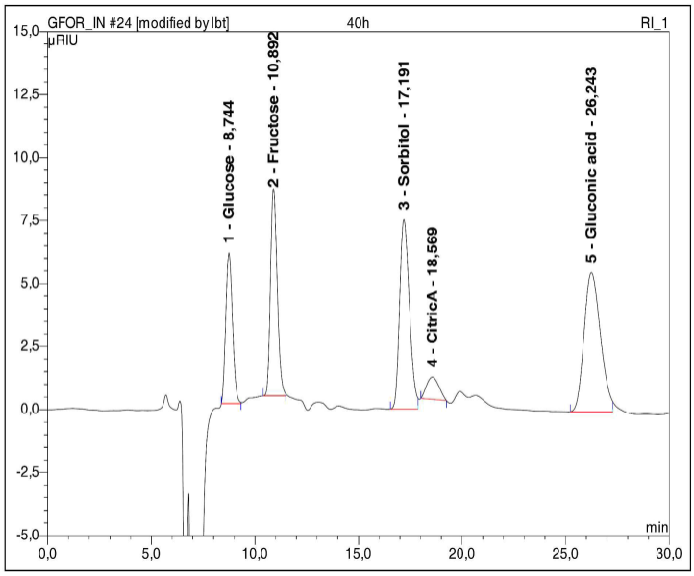
Figure 4. SDS-Ployacrylamide gel electrophoresis of crude extract and dye column purified GFOR (Lane 1 and 8: Precision Plus Protein Standards, Dual Color, Lane 2: Combined mixture of Fraction 28, 29, 30 and 31, Lane 3: Fraction 31 from yellow 7 dye column, Lane 4: Fraction 30 from yellow 7 dye column, Lane 5: Fraction 29 from yellow 7 dye column, Lane 6: Fraction 28 from yellow 7 dye column, Lane 7: Crude extract.

By contrast, for the enzyme purified from the *Z. mobilis* DSM 473 a dimeric formation was also proposed based on results of gel filtration experiments (Gollhofer, 1995). During heat shock inactivation a tetrameric conformation was determined by Fürlinger et al. (1998) and the average molecular mass was found to be 159234 D. Eelectrophoresis of fractions collected during purification on the dye column showed a band at around 40 kD, also indicating a tetrameric subunit structure.

Batch conversion

For batch conversion of pineapple juice sugar invertase was used to convert sucrose in to glucose and fructose, which increased the concentration of the glucose and fructose content in juices (Aziz et al., 2011). It was observed that the activity of inveratse and glucose-fructose oxidoreductase remained unaffected during simultaneous conversion. The effect of invertase and GFOR simultaneously on sugar conversion of pineapple juice was conducted in a batch reactor. The results are shown in Figure 4. As shown in Figures 5 (left), batch kinetics exhibited a typical sequential reaction. As sucrose hydrolysed by invertase, the amount of glucose and fructose content in fruit juice was raised and at the same time gluconate and sorbitol were gradually formed in an approximately equimolar ratio as expected. After 24 h of reaction about 7.83 g/L glucose and 8.96 g/L fructose were remain as residual sugars. There was no inhibition was observed as the residual sugars were found almost the same as that was found during separate conversion reaction. The profile of conversion is shown in chromatogram (Figure 5, right).

It is noticeable that the complete conversion of juice sugar was not achieved. As demonstrated by Zachariou and Scopes (1986), complete conversion of glucose and fructose solution was obtained by the presence of lactonase. During continuous production of sorbitol and gluconic acid by using permeabilized cells of *Z. mobilis* obtained after treatment with toluene (Chun and Rogers, 1988) or detergent (Rehr et al., 1991) and after freezing or drying, complete conversion of glucose and fructose was reported. Permeabilized cells allow the small cofactors to leak out of the cells and thus avoid significant formation of ethanol from glucose. Permeabilized cells after immobilization in alginate beads (Chun and Rogers, 1991; Roh and Kim, 1992; Kim and Kim, 1992) or in hollow fibre allowed an efficient continuous substrate conversion for more than 100 h. Nevertheless, pineapple



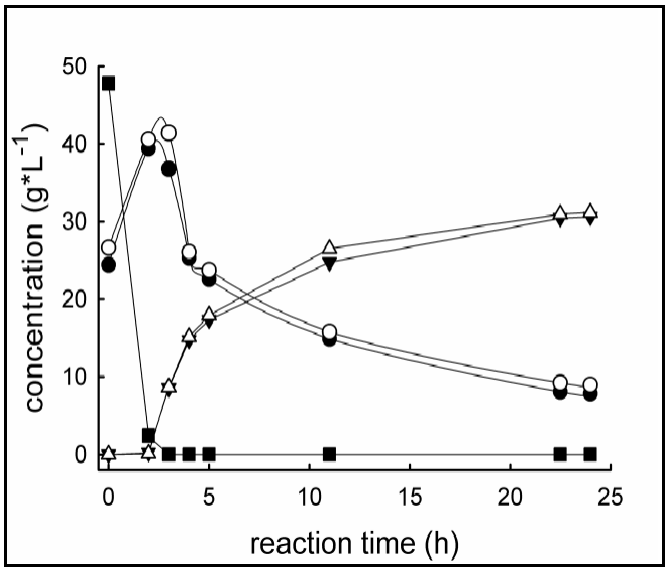


Figure 5. Time course of gluconic acid and sorbitol formation in simultaneous batch operation at 25 °C and at pH 6.2. Chromatogram given on left side showed the profile of products formation after 10 h.

juice is a complex mixture having many salts ingredients, which might affected in inhibition of conversion. It is also not unlikely that gluocose-fructose oxidoreductase as cell free may less stable than that of as permeabilized cell. Longer reaction time might also be another reason.

Conclusion

The production of glucose-fructose oxidoreductase from *Z. mobilis* was found very simple by sub-merge fermentation and could be possible to purify with minimum steps. Only one step was found enough for using in conversion experiments. Though 100% conversion of pineapple juice sugars was not achieved, however, it was found quite potential in converting juice sugars into dietetic derivatives. The major question that needs to be noted is the affect of pH due to production of gluconic acid during conversion in the sensory properties of final products. Anyway, the application of this process on juices of other tropical fruits (like papaya, jackfruit, mango) could even be more advantageous due to their higher natural pH.

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