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Evaluation of agro-food byproducts for gluconic acid production by *Aspergillus niger* ORS-4.410

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Summary

Certain cost-effective carbohydrate sources in crude as well as after purification were utilized as the sole sources of carbon for gluconic acid production using *Aspergillus niger* ORS-4.410 under submerged fermentation. Crude grape must (GM) and banana-must (BM) resulted into significant levels of gluconic acid production i.e. 62.6 and 54.6 g/l, respectively. The purification of grape and banana-must led to a 20–21% increase in gluconic acid yield. Molasses as such did not favour gluconate production (12.0 g/l) but a significant increase in production (60.3 g/l) was observed following hexacyanoferrate (HCF) treatment of the molasses. Rectified grape must (RGM) appeared to be best suitable substrate which after 144 h resulted in 73.2 g of gluconic acid/l with 80.6% yield followed by the yield obtained from the rectified banana must (RBM) (72.4%) and treated cane molasses (TM) (61.3%). Abundant growth of mould *A. niger* ORS-4.410 was observed with crude grape (0.131 g/l/h) and banana must (0.132 g/l/h).

Introduction

Gluconic acid is regarded as a bulk chemical and is used as an important product in the food, feed, beverage and textile industries and for various clinical approaches. Due to the potential demand for it of about 50,000 to 60,000 tons per annum, microbial fermentation is exclusively used for commercial scale production using glucose as a major carbohydrate source (Roehr *et al.* 1996; Roukas 2000). There are many reports on the fermentative production of gluconic acid and its salts by various bacterial and mould species. The commonly studied bacterial species belong to *Pseudomonas, Acetobacter, Gluconobacter, Zymomonas* (Bekers *et al.* 2000; Chen & Liu 2000; Moonmangmee *et al.* 2000), while in moulds, *Penicillium, Aspergillus*, *Aureobasidium* (Petruccioli 1994; Anastassiadis *et al.* 2003; Singh *et al.* 2003) have been considered suitable strains for gluconic acid production. Refined glucose, glucose syrup and sucrose have been the main substrates for gluconic acid production (Ray & Banik 1999; Silveira *et al.* 1999; Bekers *et al.* 2000). The process could be further economized by replacing conventional refined carbohydrate materials with more economical substrates. A large quantity of raw fruit materials during storage undergo decomposition and generate a waste that may cause environmental pollution. Utilization of these waste materials can be a part of environmental pollution control on one hand and production of value-added products of commercial significance on the other, thus changing their status from waste to potential provider. Agro-food byproducts such as grape-must, banana-must and sugarcane molasses contain high concentrations of sugars and can be considered as potential substrates that are easily available and economical. The present study is aimed at evaluating the economical waste carbohydrate sources grape-must, banana-must and sugarcane molasses for gluconic acid production by a mutant *Aspergillus niger* strain ORS-4.410. Concentrated purified grape-must, banana-must and treated molasses have also been evaluated for gluconic acid production.

Materials and methods

Microorganism

Aspergillus niger mutant ORS-4.410 (Singh *et al.* 2001a) derived from the wild type *Aspergillus niger* ORS-4 (ITCC 5231) (Singh *et al.* 1999) after a two step u.v. irradiation, was used for this study. The strain was maintained on potato dextrose agar (PDA) slant by periodical transfers; and was incubated following transfer for 72 h (30 °C) before storing at 4 °C.

Preparation and purification of grape-must

Grape juice has high sugar content (17% total sugar: 50% glucose and 50% sucrose) and is acidic (up to 10 g/l tartaric acid). Market-refused red grapes of the Concord variety (100% ripened) that did not meet with the quality norms were used in fermentation reaction for gluconic acid production. Clarification of grape-must was followed as described (Grassim & Fauquembergue 1996) with slight modifications. Briefly, decomposed and market-refused grapes were collected (1 kg) and mixed with 1 l double distilled water. These were then destemmed, crushed and heated at 80 °C for 30 min to release the red colour from the grape skin and to inactivate the endogenous polyphenol oxidase. Material thus obtained was filtered through muslin cloth and the juice that emerged was considered as grape-must (GM), which was then diluted to give 10–12% sugar concentration and used for gluconic acid fermentation. This grape-must was further clarified by addition of Cytolase (50 μg/g of original fruit mass) at room temperature for 30 min. The resulted free run juice was subjected to vacuum filtration, cooled at 4 °C to prevent fermentation and then depectinized with Klerzyme, (200 μM for 2 weeks). The filtrate juice was referred as rectified grape must (RGM) and diluted to 120 g glucose/l before being used for fermentation.

Preparation and purification of banana-must

Market-rejected yellow rotten bananas that did not meet quality norms for consumption was utilized as the substrate for gluconic acid fermentation. Preparation and clarification of banana-must was followed as described (Grassim & Fauquembergue 1996). Briefly, the rotten bananas (1 kg) were peeled, ground and blanched in 1 l double distilled water. The obtained slurry was heated at 85 °C for 2–3 min to inhibit polyphenol oxidase. Potassium metabisulphite (100 μM) was then added to prevent browning. The slurry was subjected to vacuum filtration and the free run juice thus collected was referred to as banana-must (BM). Further, banana-must was treated with Rapidase (75–100 μg/g of fruit pulp for 1–2 h at 45 °C) and clarified by centrifugation (5000 × g, 30 min). Clarified supernatant juice was referred as rectified banana-must (RBM) and further diluted to 120 g glucose/l of fermentation medium prior to fermentation set-up.

Clarification of molasses

Crude molasses (CM) was found to contain high concentrations of heavy metals and other compounds that inhibited gluconic acid fermentation, hence it was treated with hexacyanoferrate (HCF) prior to use. The crude cane molasses (1 kg, obtained from a local sugarcane mill) was diluted 4–5 times with deionized water and passed through a bed of activated charcoal for decolourization. HCF (3.8 mM) was added to the decolorized molasses at pH 4.0–4.5, followed by heating at 70–90 °C for 15 min. The precipitate formed containing metallic complex was removed by filtration, and the filtrate was referred as treated cane molasses (TM). The pH of clarified molasses was adjusted to 4.5 before its use for gluconic acid fermentation.

Growth and fermentation condition

The spores (5 days-old) were suspended in 5 ml of sterile 50 mM phosphate buffer (pH 6.8) containing 0.1% (v/v) Tween-80 (10^{10}–10^{12} spores/ml) and used as inoculum (2–3%, v/v) for batch fermentation. The fermentation medium contained: $(NH_4)_2HPO_4$, 1.0 g/l; KH_2PO_4, 0.5 g/l; $MgSO_4.7H_2O$, 0.15 g/l; $CaCO_3$, 40 g/l (sterilized separately), medium was supplemented with 120 g/l glucose from previously diluted each substrate type i.e. grape-must (corresponding to ~250 g t.r.c./l), RGM (corresponding to ~250 g t.r.c./l); banana-must (corresponding to ~240 g t.r.c./l), RBM (corresponding to ~240 g t.r.c./l); crude hydrolysed molasses (corresponding to ~285 g t.r.c./l) and HCF-treated molasses (corresponding to ~285 g t.r.c./l) as the sole carbon source in separate fermentation reactions. Initial pH was 5.5 at 30 °C unless $CaCO_3$ was added in the medium (pH 6.5 ± 0.1). The submerged culture cultivation was carried out in batches using Erlenmeyer flasks (500 ml), each containing 100 ml medium; flasks were incubated at 30 °C in an Orbital shaker (Sanyo Gallenkamp, U.K.) at 150 rev/min for up to 8 days.

Determination of glucose and gluconic acid

Unfermented total residual sugar was determined according to Miller (1959) and the total reducing carbohydrate was estimated as described by Mann & Saunders (1960). The gluconic acid formed was qualitatively analysed by HPLC (Waters, Milford, USA) using C18 ODS2 column. Elution was performed with an isocratic solvent (0.8 ml/min) using acetonitrile: H_2O (3:7 v/v) and detected at 210 nm. A standard solution of gluconic acid (Sigma) was prepared and eluted similarly. The elution times of peaks were compared to the elution time of a standard peak. Fermented broth containing gluconic acid was subjected to acid hydrolysis and the resulting gluconolactone was measured by a modified hydroxamate method (Lien 1959). Total yield of gluconic acid was determined by measuring the dissolved calcium in the fermentation broth as described by Lehman (1985). Briefly, 2 ml of supernatant (obtained by centrifuging fermented broth) was diluted with 600 ml of double distilled water. To this, 5 ml of concentrated ammonia solution was added followed by a pinch of Eriochrome-red B powder. The sample thus prepared was titrated with 0.1 M Titriplex solution until the color changed from yellow to green (1 ml Titriplex III = 2.004 g Ca/l broth; Total gluconic acid yield (%) = gluconic acid produced/ total sugar utilized × 100).

Determination of dry cell mass

Culture fluid was filtered through Whatman No. 1 paper. The filtered mycelia were washed with acidified (pH 2.5 with 4 M HCl) doubled distilled water to convert the insoluble $CaCO_3$ to soluble $CaCl_2$. The separated mycelia were washed several times with deionized water until pH of washing was 7.0; mycelia were then dried at 75 °C to constant weight after repeated weighing.

Reproducibility of results

All fermentation was carried out in triplicate and the experimental results represent the mean of three identical fermentations. Statistical analysis was performed using ANOVA test software.

Results and Discussion

Utilization of grape-must and the banana-must for gluconic acid fermentation

Cheap carbohydrate sources such as GM, RGM, BM and RBM and cane molasses were evaluated for gluconic acid production by *A. niger* mutant ORS-4.410. Among all the substrates used, RGM appeared to be a potential substrate resulting into higher levels of gluconic acid. An increase in the levels of gluconic acid produced with RGM was observed after 72 h followed by maximum production (73.2 g/l) after 144 h (Figure 1) with 80.6% yield, whereas, GM resulted into 62.6 g gluconic acid/l, with 60.4% yield. Yield was

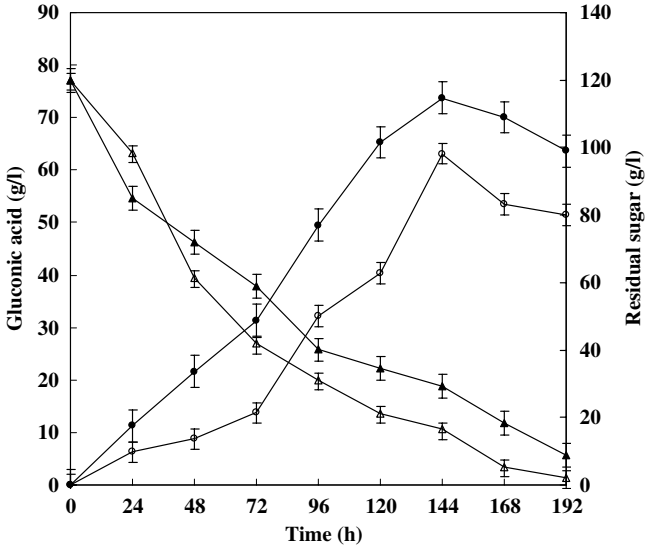

Figure 1. Utilization of grape-must (○, △) and rectified grape-must (●, ▲) as a sole source of carbon for gluconic acid production (○, ●) and residual sugars (△, ▲) by *Aspergillus niger* ORS-4.410. The fungus was grown in fermentation medium under submerged culture cultivation with an initial pH of 6.5 at 30 °C.

therefore 20% lower with GM as compared with that of RGM as the substrate. ANOVA test for significant differences between gluconic acid production was performed at different time periods for both substrates (GM: $F=103.4$; d.f. = 7, 16; $P=0.000$; RGM: $F=135.4$; d.f. = 7,16; $P=0.000$). Kinetic analysis of the bioconversion also showed that the degree of conversion (0.611 g/g) and gluconic acid production rate (0.509 g/l/h) were higher with RGM as compared to the GM (degree of conversion, 0.522 g/g; gluconic acid production rate, 0.435 g/l/h) (Table 1). *A. niger* mutant ORS-4.410 yielded rapid growth on the crude substrates such as grape-must and banana-must and had shown higher substrate utilization (Table 1) but had lower gluconic acid productivity. Significant growth of mould *A. niger* ORS-4.410 was observed during 24–96 h of fermentation with GM having a specific biomass growth rate of 0.131 g/l/h whereas a lower growth rate (0.106 g/l/h) was observed with RGM (Table 1). Higher salt concentration in the crude substrates i.e. GM and BM (1.0 and 1.9 g total nitrogen/l, respectively) (Holland *et al.* 1997) may possibly favour biomass accumulation than the gluconic acid accumulation (Buzzini *et al.* 1993; Ray & Banik 1999). Grape and banana-must when rectified appeared to be the better substrates that has improved gluconic acid yield 20–21% in fermentation medium. The biosynthetic activity of *A. niger* ORS-4.410 increased rapidly after a latent period of 24 h followed by the maximum production after 144 h, acid production activity of the mould afterwards declined, probably due to the reduced amount of glucose in the fermentation medium.

Another cheaper substrate i.e. RBM from market-refused banana was evaluated for gluconic acid production. Use of banana-must as such led to 54.6 g/l gluconic acid, while RBM was found to result into a 27% increase in acid production (Figure 2) with significant yield 72.4% as compared to crude banana-must (51.7%) (Table 1). A total of 79.6 and 88.0% of the glucose were utilized after single cycle of fermentation (144 h) from RBM and BM, respectively (Table 1). Significant differences in between gluconic acid production were found at different time intervals using RBM and BM as sole carbon sources in fermentation medium (BM: $F=76.64$; d.f. = 7, 16; $P=0.00$; RBM: $F=231.2$, d.f. = 7, 16; $P=0.000$).

Utilization of sugarcane molasses for gluconic acid fermentation

Molasses resulted into lower levels of gluconic acid production (12.0 g/l), however a notable increase in gluconic acid production was observed with TM (60.3 g/l) when used as the substrate (Figure 3). Comparison of gluconic acid yields as obtained from TM (61.3%) with that of rectified fruit wastes (RGM, 80.6% and RBM, 72.4%) had indicated that TM had yielded a comparatively lower amount of gluconic acid

Table 1. Evaluation of the kinetic parameters for gluconic acid production using cheap carbohydrate sources by *Aspergillus niger* ORS-4.410

Parametersa[a]	GM	RGM	BM	RBM	CM	TM
Gluconic acid yield[b] (%)	60.4	80.6	51.7	72.4	15.3	61.3
Degree of conversion[c] (g/g)	0.522 (±0.241)	0.611 (±0.306)	0.455 (±0.211)	0.578 (±0.293)	0.100 (±0.098)	0.486 (±0.251)
Gluconic acid production rate[d] (g/l/ h)	0.435 (±0.219)	0.509 (±0.301)	0.379 (±0.127)	0.481 (±0.139)	0.083 (±0.021)	0.405 (±0.206)
Specific glucose uptake rate[d] (g/l/ h)	0.721 (±0.302)	0.631 (±0.264)	0.733 (±0.348)	0.115 (±0.095)	0.083 (±0.026)	0.118 (±0.094)
Glucose utilization[e] (%)	86.5	75.8	88.0	79.6	65.3	79.3
t.r.c. utilization (%)	57.5	75.4	61.7	66.1	49.3	59.4
Specific biomass growth rate[d] (g/l/ h)	0.131 (±0.093)	0.106 (±0.084)	0.132 (±0.075)	0.115 (±0.094)	0.083 (±0.031)	0.118 (±0.071)
Biomass yield[d, f] (g/g)	0.183 (±0.086)	0.168 (±0.076)	0.180 (±0.073)	0.174 (±0.085)	0.153 (±0.062)	0.178 (±0.079)

[a] Analyzed at 144 h of fermentation.
[b] Calculated as per utilized glucose.
[c] Degree of conversion (1 = p/s), P, product; S, initial glucose concentration.
[d] Mean of three replicates ± S.D.
[e] Calculated as per utilized total reducing carbohydrate (t.r.c.).
[f] Y(g/g), values calculated on the basis of biomass obtained and the substrate utilized.

than the other two rectified fruit wastes used (Table 1). Statistically, significant differences were found in between gluconic acid production using crude and TM (CM: $F = 2.254$; d. f. = 7, 16; $P = 0.085$; TM: $F = 125.8$; d.f. = 7, 16; $P = 0.000$). The higher concentrations of heavy metal ions not only hindered gluconic acid production but also sustained the cellular growth of the mould. The reduction in gluconic acid production may be due to the inactivation of intracellular glucose oxidase at higher metal ion concentration (Liu *et al.* 2001). Utilization of total reducing carbohydrates was lower with CM when compared to the other two substrates GM and BM (Table 1).

It is therefore apparent that RGM was the better carbon source resulting in 6 and 22% higher production of gluconic acid than RBM and treated molasses, respectively. The analysis of gluconic acid production by *A. niger* mutant ORS-4.410 indicated that direct fermentation of pure glucose resulted in 91.7 g gluconic acid/l, with 94.5% yield after 144 h of incubation (Singh *et al.* 2001b). Comparison of total gluconic acid production from RGM, RBM and TM with glucose indicated that RGM, RBM and TM resulted in 80, 76 and 66% gluconic acid production with respect to the production obtained with glucose. These observations therefore substantiated that the rectified grape, banana-

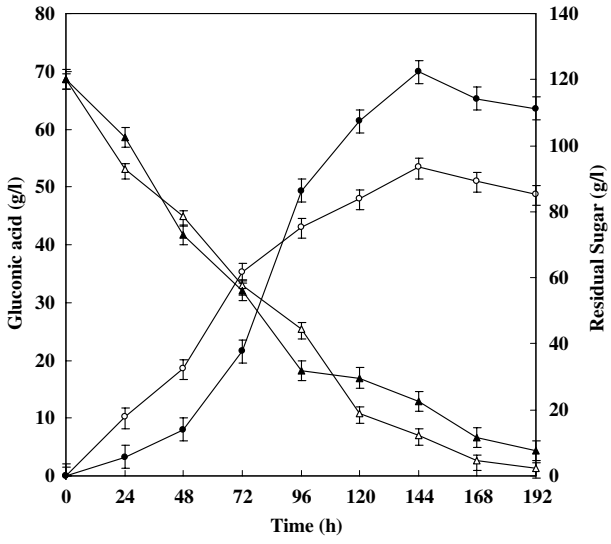

Figure 2. Utilization of banana-must (○, △) and rectified banana-must (●, ▲) as a sole source of carbon for gluconic acid production (○, ●) and residual sugars (△, ▲) by *Aspergillus niger* ORS-4.410. The fungus was grown in fermentation medium under submerged culture cultivation with an initial pH of 6.5 at 30 °C.

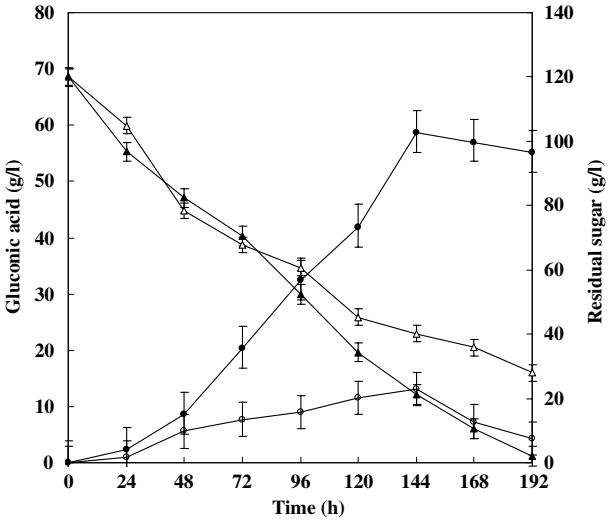

Figure 3. Production of gluconic acid (○, ●) and recovery of residual sugars (△, ▲) in fermentation medium using crude (○, △) and HCF treated cane molasses (●, ▲) under submerged fermentation by fungus *Aspergillus niger* ORS-4.410, grown in fermentation medium with an initial pH 6.5 at 30 °C.

must and the treated molasses are potential substrates for gluconic acid production. Depending upon the substrate used, varying degree of the unfermented sugars remained in the fermentation medium and consisted mainly of complex carbohydrates, which were the major carbohydrate residual material of each substrate. Earlier, attempts have been made to utilize the fig, grape-must and sugarcane molasses for gluconic acid production using *Aspergillus niger* and *Penicillium funiculosum* MN 238 strains (Kundu & Das 1984; Buzzini *et al*. 1993; Roukas 2000); however, *A. niger* ORS-4.410 appeared to have yielded higher production levels with RGM and treated cane molasses as compared to the earlier reports (Kundu & Das 1984; Buzzini *et al*. 1993). Analysis of kinetic parameters had also clearly demonstrated RGM followed by RBM are the potential raw substrates for gluconic acid production (Table 1). Present study thus reveals that mutant *A. niger* ORS-4.410 can be an effective and promising mould that could be utilized for gluconic acid production using horticultural and agricultural byproducts as the cheaper carbohydrate substrates.

Economics is of prime importance for any fermentation industry to be viable and successful, and to a greater extent depends on selection of the materials for the process. The choice is undoubtedly for the more economical carbohydrate raw materials provided that the microorganisms do not impose any special requirements for the particular substrate. These specificities, therefore, led to the search for significant carbohydrate materials containing high sugar content and that are wastes with no further applications. Fruit wastes are generated either as decomposed fruit pulps during storage and processing of the fruit material in horticulture industries or as market-rejected fruit wastes from numerous regional markets. These wastes are easily available in market at substantially lower prices or at free of cost. Among the fruit wastes, grape-must and the banana-must appear to have a high sugar content. In addition, the molasses that are generated from the sugarcane processing industries do have the high sugar content and are also low priced. These materials, which are cost-effective and are easily available, therefore, are promising substrates for economical production of this industrially significant product.

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