Utilization of Agro-food By-products for Gluconic Acid Production by Aspergillus niger ORS-4 Under Surface Culture Cultivation

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Among the seven isolated microbial strains from dumping sites of the sugarcane industry waste, a potent fungal strain Aspergillus niger ORS-4 was selected, that gave 48 g/L of gluconic acid with 74 per cent yield when glucose was used as the carbon source. Starch hydrolysate, molasses and the banana must were evaluated as the cheaper carbohydrate sources for gluconic acid production by A. niger ORS-4 in surface culture fermentation process. The banana must was found to be a better source with significant gluconic acid production (39.6 g/L, 40 per cent yield) after 12 d incubation. The untreated sugarcane molasses gave marginal production of gluconic acid (2.4 g/L), however, the production increased significantly (34.6 g/L, yield 38.5 per cent) after the molasses were subjected to the hexacyanoferrate (HCF) treatment. Starch hydrolysate on the other hand, resulted into comparative production (30.2 g/L, yield 35.9 per cent) but lower than that obtained with HCF treated molasses, whereas the acid production was low (10 g/L) with unhydrolyzed starch. Gluconic acid production from these substrates was comparable to that obtained with glucose.

Introduction

Gluconic acid is an oxidative product of D-glucose. Due to its low toxicity, low corrosivity and complexing abilities with divalent and trivalent metal ions the gluconic acid has found its extensive use in the pharmaceutical, food and leather industries. Thus, the demand for gluconic acid is about 50,000-60,000 t/y and still growing year after year. It led many groups to analyze and select an effective system for increased and economical production of gluconic acid. Among the various substrates, glucose, glucose syrup and sucrose are most commonly used substrates. The process of gluconic acid production can be further economized by using the cheaper carbohydrate sources for acid production. Efforts have been made to utilize the by-product of agricultural industry e.g., molasses, starch hydrolysate and concentrated rectified grape must as substrates for the gluconic acid production. Suitable processing of the substrates and appropriate strain selection may lead to a further improvement in the gluconic acid production.

The present study has been directed to evaluate bioconversion of horticultural waste, i.e., banana must, agricultural industry by-product like molasses and the starch as substrates for gluconic acid production by a potent strain of Aspergillus niger ORS-4 in surface culture process. The banana must, molasses and the corn starch are easily available and are relatively low-priced, however, the presence of heavy metal ions and the complex carbohydrate such as starch may be deterrents towards utilizing the substrate for the production of gluconic acid and require suitable treatments before their utilization for the acid production.

Materials and Methods

Microorganism

The microbial strain A. niger ORS-4, used in this study, was isolated from the dumping sites of sugarcane industry wastes. The strain was maintained on potato dextrose agar (PDA) slants by periodical transfer at 30 °C for 72 h before storing at 4 °C.

Preparation of Banana must

The market-refuge ripened bananas that did not meet the quality norms were utilized as a cheap
carbohydrate substrate for gluconic acid fermentation. The fully ripened, peeled, ground and blanched banana juice was clarified as described by Grassi and Fouquenbergue. Briefly the extracted juice was added with potassium metabisulphite at 85 °C to prevent browning of banana juice. After filtration and centrifugation, the clear juice obtained was diluted to contain 12 per cent reducing sugars and used as the substrate for the fermentation media.

**Clarification of Molasses**

The sugarcane molasses were diluted 3-4 fold and then clarified by hexacyanoferate (HCF) treatment. The clear supernatant was diluted to contain 12 per cent reducing sugars and used as substrate for fermentation.

**Preparation of Starch Hydrolysate**

Commercial corn starch was hydrolysed and liquefied with HCl. Starch slurry (20 per cent) was prepared in water, pH adjusted to 2 with 1N HCl and autoclaved for 30 min at 10 psi. The hydrolyzed starch was cooled and neutralized with CaCO₃ followed by filtration. The filtrate was diluted to contain 12 per cent reducing sugars and used as substrate for fermentation.

**Fermentation media**

The fermentation medium in separate sets utilized glucose, banana must, clarified molasses and starch hydrolysate as carbohydrate source in concentrations equivalent to 12 per cent reducing sugar (glucose). The salt mixture containing (NH₄)₂HPO₄ 0.1, KH₂PO₄ 0.05, MgSO₄.7H₂O 0.015 and CaCO₃ 4.0 per cent was added in the media sterilized separately and then added to the fermentation medium before inoculation.

**Fermentation**

Fermentation was carried out in Erlenmeyer flask (500 mL) containing 50 mL medium. The inoculum was prepared from aseptically harvested spores of the subcultured Aspergillus niger ORS-4, at 30 °C for 5 d and suspended in 5 mL sterile 0.05M phosphate buffer (pH 6.8) containing 0.1 per cent Tween 80 (representing 10¹⁰-10¹² spores/mL). The spore suspension was used as inoculum at 2-3 per cent level for the biocconversion process. The surface culture cultivation was performed in batches at 30 °C for the desired time period under proper aeration.

Waste fermentation gases were removed through water suction pump.

**Determination of gluconic acid and glucose**

The total reducing carbohydrate was determined by Fehling method as described by Mann and Sounders and the unfermented residual sugar was determined according to Miller. The gluconate formed and yield of gluconic acid was determined via the dissolved calcium amount in fermentation broth as described by Lehmann. The broth containing gluconic acid was subjected to acid hydrolysis and resulting gluconolactone was measured by modified hydroxamate method.

**Determination of Dry Cell Mass**

The culture fluid was filtered through a Whatman No. 1 filter. The filtered mycelia were acidified (to pH 2.5 with 4N HCl) in order to convert insoluble CaCO₃ to soluble CaCl₂. The separated mycelia were washed several times with deionized water until the pH of the washing was 7.0. The mycelia then were dried at 75 °C to a constant weight.

**Reproducibility of Results**

All fermentation reactions were carried out in triplicate and the mean results have been reported.

**Results**

The agro-food by-products e.g., banana must, molasses and the starch hydrolysate were evaluated for gluconic acid production by the strain A. niger ORS-4 isolated from the sites decomposed with sugarcane industrial wastes. Among cheaper substrates used, highest production of gluconic acid was obtained with banana must. Rate of production was lower up to four days of incubation followed by an exponential increase leading to the maximum production (39.60 g/L) with 40.08 per cent yield after 12-14 d incubation (Figure 1). A similar pattern was observed for biomass accumulation.

The equivalent amount of the cane molasses on the other hand produced 34.56 g/L of gluconic acid with 38.47 per cent yield after 12-14 d incubation (Figure 2). Untreated cane molasses yielded marginal production of the gluconic acid than the treated molasses. Comparatively lower acid production (30.12 g/L) with 35.9 per cent yield was obtained with starch hydrolysate (Figure 3). Therefore, comparison of gluconic acid production with different
substrates showed 4 and 11 per cent higher yield with banana must as compared to treated molasses and the hydrolyzed starch, respectively. It was further evident through 82.5 per cent utilization of sugar with banana must, the highest, compared to 75 and 70 per cent utilization with treated molasses and hydrolyzed starch respectively (Figure 1-3). Various kinetic parameters for gluconic acid production by A niger ORS-4 were analyzed. Maximum rate of gluconic acid production, 3.30 g/L/d, was observed with banana must followed by the treated molasses and the starch hydrolysate with 2.88 and 2.51 g/L/d, respectively. Similarly the glucose uptake rate with banana must (8.25 g/L/d) was higher than the other substrates used (Table 1). However, higher specific growth rate of 1.10 g/L/d of A niger ORS-4 was observed with treated molasses and it accounts for factors and remaining salts present in treated molasses which supported more vigorous growth of the organism, also affecting the acid production to some extent. The gluconic acid production by A niger ORS-4 was also determined after direct fermentation of pure glucose that resulted in a higher accumulation of gluconic acid (48.48 g/L) with 70-75 per cent yield after 12 d incubation.

**Discussion**

In the industrial process, economics is of prime importance and to a greater extent it depends on the selection of raw materials. The choice undoubtedly is among the cheapest raw materials provided that the microorganisms do not have any special requirements for particular carbohydrates. It has lead to widespread use of molasses in fermentation industries. Starch has also found wide applications as the carbohydrate sources especially in the preparation of amylolytic enzymes. Among the carbohydrate sources with high sugar content and that are easily available as the waste material by-products during storage and processing, the banana must and the...
molasses appear as promising substrates for fermentation reaction. The strain Aspergillus niger ORS-4 was found to grow well on the crude substrates, particularly banana must and molasses, showing the tolerance of the strain to higher salt and heavy metal ion concentrations. Substrates appeared to be utilized under these conditions but acid production observed was lower. Higher biomass accumulation with molasses and banana must may be due to the higher salt and the nitrogen contents (1.0–1.9 g/L total nitrogen) in the respective substrates.

Although attempts have been made to utilize the starch for gluconic acid production, the biosynthetic activity of A niger ORS-4 increased rapidly after a latent period of 4–6 d for gluconic acid synthesis when the starch hydrolysate rather than unhydrolyzed starch was used as the substrate. The activity of the mold slowed down afterwards probably due to the exhaustion of glucose in the medium. It may be inferred that polysaccharides, irrespective of the cheaper carbohydrate materials used as the substrate, are the major residual material that may lead to a decreased yield during acid production and similar trend has been depicted in Figure 4. The various kinetic parameters analyzed (Table 1) indicated that the banana must followed by treated molasses and the starch hydrolysate are the respective potential raw substrates yielding significant levels of gluconic acid. The production of gluconic acid obtained with the raw materials is comparable with that obtained with glucose as the substrate.

The present study, therefore, demonstrates that agricultural by-products and horticultural waste material can be potentially utilized for the gluconic acid production by A niger ORS-4. The production can be effectively increased after suitable treatments or hydrolysis of the substrate starches. The strain Aspergillus niger ORS-4, therefore, appears to be a promising strain for industrial scale fermentation of the gluconic acid using cheap carbohydrate sources. Further work is in progress to identify other critical parameters and efforts are also directed to derive mutants for improving and economizing the process for gluconic acid production.

References


