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# UTILIZATION OF GRAPE MUST AND CONCENTRATED RECTIFIED GRAPE MUST TO PRODUCE GLUCONIC ACID BY ASPERGILLUS NIGER, IN BATCH FERMENTATIONS.

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## SUMMARY

Grape must and concentrated rectified grape must were used for the gluconic acid synthesis using Aspergillus niger batch cultures. The latter substrate was the better, with a production, at 72 h, of 67.43 g/l and a yield (calculated on converted glucose) of 0.96. Citric acid was also observed as a by-product. In order to decrease the residual fructose content, at the end of the gluconate production cycle, an experimental model of sequential fermentation A. niger - Rhizopus arrhizus was proposed for the synthesis of gluconic and fumaric acid. The use of Glucose-isomerase (EC 5.3.1.5) to convert fructose to glucose was also tested.

## INTRODUCTION

The utilization of glucose as carbon source for gluconic acid production by Aspergillus niger nowadays seems to be uneconomical (Roukas and Harvey, 1988). Agro-food by-products and surplus were already considered as cheaper carbohydrate sources for this purpose (Roukas and Harvey, 1988; Buzzini et al, 1992a; Buzzini et al, 1992b) and to produce citric (Rossi et al, 1988) and fumaric acids (Buzzini et al, 1991; Gobbetti et al, 1991; Parente et al, 1992). Grape must can be considered a suitable substrate being easily available and relatively low priced. However, its use by A. niger sometimes presents problems for the inability to utilize fructose (which is present together with glucose in almost equivalent amounts) for the gluconic acid synthesis (Murtaugh and Mahieu, 1960).
In the present work we want to point out the preliminary results with regards to the comparisons between the gluconic acid productions by A. niger on grape must as such and on concentrated rectified grape must. It was also proposed an experimental model of sequential fermentation A. niger - Rhizopus arrhizus for the utilization of the residual fructose in the medium at the end of the gluconate production cycle, as it was already made for Gluconobacter oxydans on the same substrates (Buzzini et al, 1992a). Alternatively, the use of immobilized Glucose-isomerase (EC 5.3.1.5) to convert fructose to glucose was also tested.

## MATERIALS AND METHODS

Microorganisms and cultural conditions. Aspergillus niger ATCC 60363 was

grown on slant of malt agar medium and stored at 5°C. A spore suspension ($2x10^8$/ml) was used to inoculate (5% v/v) the precultures (Rohr et al, 1983). 130-150 pellets were utilized, together with 3 ml of preculture, for the seeding (12% v/v) of the production media.

Substrates. Concentrated rectified grape must (CGM, 980 g/l of total reducing carbohydrates - t.r.c., undetectable nitrogen content) diluted until 70 g/l of glucose (corresponding to $\sim$ 140 g/l of t.r.c.) and integrated with 0.7 g/l of $(NH_4)_2HPO_4$; grape must (GM, 70 g/l of glucose, $\sim$ 140 g/l of t.r.c., 0.75 g/l of nitrogen); glucose syrup (GS, 790 g/l of equivalent glucose - e.g., after acid hydrolysis, 4.65 g/l of nitrogen, SPAD, Alessandria, Italy) diluted to 140 g/l of e.g., were used. 0.19 g/l of $KH_2PO_4$, 0.16 g/l of $MgSO_4.7H_2O$, 0.8 g/l of $MnSO_4.4H_2O$ were added to CGM and GS. 20 g/l of $CaCO_3$ (as neutralizing agent) were also added to all the substrates. The pH was then adjusted, before the sterilization, by NaOH 2 N (final value 6.0-6.5).

Batch fermentation. The submerged fermentations were carried out in 500 ml shaken flasks by utilizing a heated agitator (Adolf Kuhner Lab-shaker + Lab-therm, Mod. LT-V) and controlling the following parameters: substrate-flask volumetric ratio 1:20, 180 rpm, 30°C.

Sequential fermentation. CGM (diluted until 280 g/l of t.r.c. and integrated with the above mentioned salts) was employed for the growth of *A. niger*. After 72 h, the mycelium was separated (by centrifugation, 10,000 rpm for 10 min) and the medium was reintegrated with 1 g/l of $(NH_4)_2HPO_4$, 0.05 g/l of $ZnSO_4.7H_2O$ and 40 g/l of $CaCO_3$. The pH was corrected by sterile NaOH 2 N (final value 7.0). 20-25 pellets of *Rhizopus arrhizus* ATCC 13310, obtained as previously described (Buzzini et al, 1991), were used to inoculate the substrate.

Use of Glucose-isomerase. CGM (diluted until to 280 g/l of t.r.c. and integrated with the above mentioned salts, except for $CaCO_3$) was used for the gluconic acid synthesis by *A. niger*. 0.4 g/l of $CaSO_4.2H_2O$ was also added. The pH was periodically controlled by the addition of sterile NaOH 2 N. After 72 h, the pH was again corrected to 6.5 and Sweetzyme T (immobilized Glucose-isomerase EC 5.3.1.5, Novo Nordisk, Milano, Italy) was added (12.5% w/v) to the substrate (Novo-Nordisk, 1992). The pH was readjusted every 12 h.

Analytical methods. After each fermentation cycle, KOH was added to the samples (Moellering and Bergmeyer, 1984), to obtain the hydrolysis of D-glucono-$\delta$-lactone. Glucose, gluconic, fumaric and citric acids were determined by a modified (Buzzini et al, 1992a) gas-chromatographic method (Damiani and Bartocci Pandolfi, 1973). Gluconic acid, glucose and fructose contents on cultural broths were also controlled with enzymatic method (Moellering and Bergmeyer, 1984; Kunst et al, 1984; Beutler, 1984). Other parameters were determined as follows: t.r.c. by the Fehling method, nitrogen content of the substrates and produced biomass (as insoluble nitrogen), by the semi-micro Kjeldahl method (Kjeltec System PBI).

## RESULTS AND DISCUSSION

Comparisons among different substrates. A decrease of pH was observed

during the fermentations until the level of 3-4. Where not already specified, the data refer to fermentation cycles of 72 h and the gluconic acid yields -Yg- are calculated on the converted glucose. As previously reported (Rohr et al, 1983), the gluconic acid accumulation by A. niger is strongly affected by various experimental conditions. The carbohydrate and nitrogen contents were previously optimized on CGM, taking into consideration that, by increasing the nitrogen (from 150 to 300 mg/l) and carbohydrate (from 140 to 280 g/l of t.r.c.) levels, a higher biomass development (from 0.45 to 0.71 g of insoluble $N_2$/l) and a reduction of the yield (from 0.96 to 0.60) were respectively observed (unpublished data).

The productive potentiality of CGM and GM for gluconic acid synthesis was compared. For both the substrates (Fig.1), the highest production was observed at 72 h (67.43 g/l and 55.42 g/l for CGM and GM respectively). By observing these results we can point out that, differently from other organic acid synthesis (Buzzini et al, 1991) CGM was found to be a more appropriate medium when compared with GM (Yg = 0.97 rather than 0.81). Another acid, such as citric acid, was also found as a by-product on cultural broths at the end of the fermentation cycle (4.02 g/l and 1.11 g/l for CGM and GM respectively). The above reported differences are related to the chemical compositions of the media (principally nitrogen content) that affect the mycelial growth and, in the opposite way, the gluconic acid synthesis. In both the media, an initial phase of progressive increase of the biomass (Fig.1) was in fact observed, but it was very short on CGM (within 12 h) and until to 72 h in GM. The nitrogen exhaustion of the substrates, in fact, occurred between 12 and 24 h for CGM (Fig.1), while a very small amount of nitrogen was still measured on GM at 72 h. The fermentation parameters observed on CGM are reported on Table 1.

Table 1: Fermentation parameters on CGM (70 g/l of glucose corresponding to ∼140 g/l of t.r.c.).

| Parameters | 12 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|
| gluconic acid yield | 0.59 | 0.95 | 0.96 | 0.96 |
| gluconic + citric acid yield (1) | 0.59 | 0.64 | 0.70 | 0.64 |
| glucose conversion (%) | 2.71 | 10.04 | 67.44 | 99.29 |
| t.r.c. utilization (%) | 1.40 | 7.88 | 50.57 | 76.36 |
| gluconic acid production rate (g/l.h) | 0.09 | 0.41 | 1.60 | 0.89 |
| glucose conversion rate (g/l.h) | 0.16 | 0.43 | 1.67 | 0.93 |
| t.r.c. utilization rate (g/l.h) | 0.17 | 0.75 | 2.49 | 1.50 |

(1) calculated on utilized t.r.c.

A latence phase (about 12 h) for the gluconic acid synthesis (% of glucose and t.r.c. conversion of 2.71 and 1.40 respectively) was observed. After this time, the biosynthetic activity of A. niger increased very rapidly, reaching the maximum values between 24 and 48 h

(gluconic acid production and glucose conversion rates of 1.60 g/l.h and 1.67 g/l.h, respectively). Afterwards, the activity of the mould slowed down, (gluconic acid production and glucose conversion rates of 0.89 g/l.h and 0.93 g/l.h, respectively) probably as a consequence of the exhaustion of glucose on the medium. At 48 h, in fact, the glucose was utilized to a great extent (67.44%). The glucose conversion was almost complete at 72 h (99.29%) while the t.r.c. utilization remained at a lower value (76.36%). The unused sugars were composed principally (98%) of fructose, which represents the most important residual carbohydrate of CGM, after that the gluconate production cycle was accomplished. This strain of A. niger did not utilize this sugar for the gluconic acid synthesis, but only for the mycelial growth and for metabolite production, like citric acid, other than gluconic acid (0.38 g of insoluble $N_2$/l and 7.13 g/l of citric acid were in fact obtained at 168 h on a medium containing only 70 g/l of fructose as carbon source - unpublished data). A decrease of fructose concentration was observed only after 12-24 h (differences between t.r.c. and glucose conversion rates of 0.01 and 0.32 g/l.h after 12 and 24 h respectively), and it rose to its maximum utilization at 48 h (0.82 g/l.h). The citric acid was observed only after 24 h, probably as a consequence of the pH decrease (below 4.0), as previously reported (Roukas and Harvey, 1988). The citric acid production rate increased until its highest value (0.12 g/l.h) which was observed between 24 and 48 h. The gluconic acid synthesis by A. niger on CGM was also compared with the results obtained on GS (Fig.2). A higher accumulation (73.39 g/l), but with a very much lower yield (0.59) and a higher mycelial growth (4.37 g of insoluble $N_2$/l) were observed at 72 h. The highest gluconic acid synthesis (82.60 g/l was obtained at 144 h, while the glucose content decreased to a very low value (0.2 g/l).

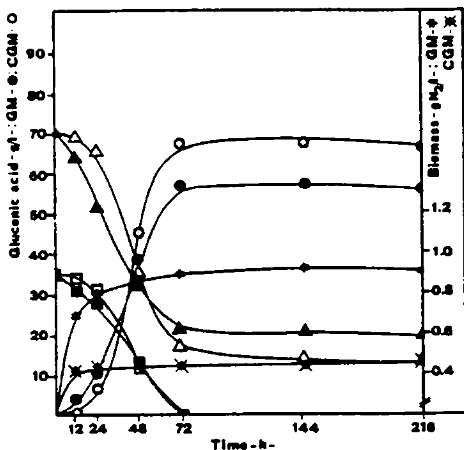


Fig. 1 Time course of gluconic acid production by A. niger on GM and CGM.

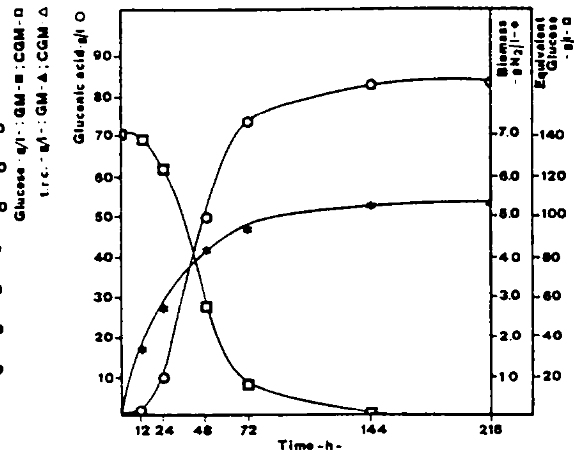


Fig. 2 Time course of gluconic acid production by A. niger on GS.

Sequential fermentation: A. niger - R. arrhizus. An experimental model of sequential utilization of A. niger and R. arrhizus was proposed for

the production of gluconic and fumaric acids to decrease the residual fructose level. In order to obtain a higher residual fructose content on the medium and higher fumaric acid productions, an initial level of t.r.c. of 280 g/l (corresponding to 140 g/l of glucose), was used (Fig. 3). After an initial growth phase of A. niger (72 h) (gluconic acid production = 85.18 g/l, Yg = 0.61, gluconic acid production rate = 1.18 g/l.h), the mycelium was separated from the substrate. Rhizopus arrhizus was then inoculated on the residual medium (t.r.c. = 80.0 g/l, glucose = 0.3 g/l after reintegration with nitrogen and salt constituents. The maximum fumaric acid production (15.59 g/l) was observed at 216 h, afterwards its level remained constant. The final concentration of t.r.c. decreased, in the same amount of time, to the value of 16.7 g/l (21% of the initial concentration). The fumaric acid production rate and yield (calculated on utilized t.r.c.) was 0.11 g/l.h and 0.25 respectively.

Use of Glucose-isomerase. Glucose-isomerase (EC 5.3.1.5 D-xylose ketol-isomerase) catalizes the reversible reaction between an aldo-pentose to its corresponding keto-isomer (Takasaki, 1967). The strategy of this experiment was to use this enzyme in the opposite direction rather than that commonly employed (glucose $\longrightarrow$ fructose) through the continuous conversion of glucose to gluconic acid by A. niger, in order to decrease the residual fructose content on the cultural broth. After 72 h of fermentation on CGM (85.42 g/l of gluconic acid, 0.7 g/l of glucose and 88.89 g/l of t.r.c.), the enzyme was added on cultural broth (Fig. 4). Only a limited increase of gluconic acid (17.17 g/l) and a decrease of residual t.r.c. content to 61.6 g/l were observed at 120 h, remaining almost constant afterwards. These results are probably the consequence of the very limiting fermentation conditions (pH, temperature), rather than those required (Takasaki, 1967; Cheng, 1983; Novo-Nordisk, 1992) for a high activity of the enzyme.

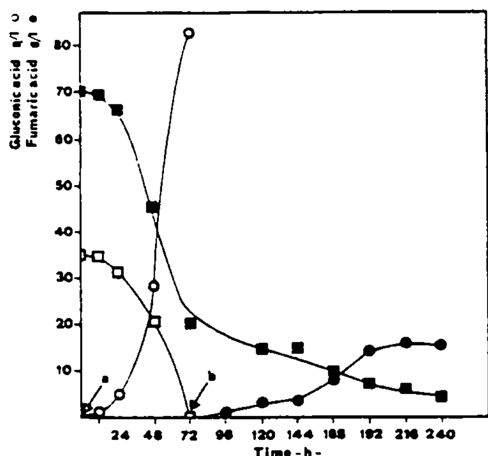


Fig. 3 Time course of gluconic and fumaric acid production by A. niger and R. arrhizus on CGM.
a = seeding of A. niger
b = seeding of R. arrhizus

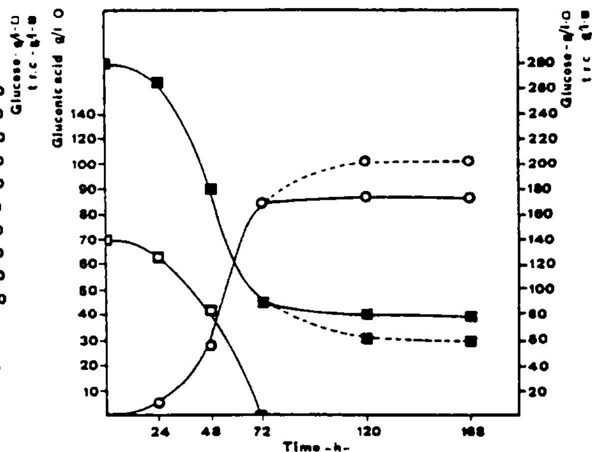


Fig. 4 Time course of gluconic acid production by A. niger on CGM. (○- - - -○) = after enzyme addition; (○——○) = no enzyme addition.

## CONCLUSIONS

Concentrated rectified grape must seems to be a suitable raw substrate for gluconic acid production by A. niger. The residual fructose content can be usefully utilized for fumaric acid synthesis, which results to be preferable in comparison to the use of immobilized Glucose-isomerase, but needs to be explored further.

## ACKNOWLEDGEMENTS

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