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Full Length Research Paper

Use of nitrous acid mutant of *Aspergillus niger* for citric acid production from local cane-molasses

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In this study, the enhancement of citric acid production from local molasses was attempted by mutagenesis. A local stain of *Aspergillus niger* was isolated from lemon leaves and treated with nitrous acid. The obtained mutant and the wild type of *A. niger* were separately used for the bioconversion of the local molasses into citric acid. The molasses fermentation was carried out at 30°C in a stirred flask. The results showed that the mutant exhibited an increased efficiency for citric acid production when compared with the wild type. It produced 230.45 gl^{-1} of citric acid; two fold increase over the parent strain (105.67 gl^{-1}), with higher sugar consumption by the mutant compared with the wild type. The maximum citric acid production levels were reached on the 9th day of fermentation by the mutant and the 10th day of fermentation by the wild strain. The mutant proved to be appropriate for citric acid production and waste material valorization in Rwanda. Also, this investigation showed itself to be one of ecofriendly technologies for Rwanda. So, the use of this technology should have impact on both the Rwanda economic development and environmental protection.

Key words: Rwanda, *Aspergillus niger,* bioconversion, cane-molasses, citric acid, mutagenesis, mutant, nitrous acid.

INTRODUCTION

Citric acid ($CH_2COOH.COH.COOH.CH_2COOH$) is ubiquitous in nature and exists as an intermediate in the citric acid cycle when carbohydrates are oxidized to carbon dioxide (Rajoka et al., 1998). Because of its high solubility, palatability and low toxicity, it can be used in food, biochemical and pharmaceutical industries. It is needed for savor reinforcement, conservation, inhibition of oxidization, carbonation maintenance, neutralization of residual washing, etc (Prescott et al., 1981; Rohr et al., 1983). These uses have placed greater stress on increased citric acid production and search for more efficient fermentation process. The worldwide demand of citric acid is about 6.0×10^5 tons per year and it is bound to increase day by day (Ali et al., 2001). Various processes are used for citric acid production. Even though the surface culture process is still being used, it has been shown that it is not very encouraging because of its very low output of citric acid (Lepoivre, 2003). The importance of this problem gave rise to many researches which, since 1940s, permitted the set up of new methods one of which is the submerged fermentation process which improved the production of the acid. In general, the process of citric acid production through the microbial fermentation established itself as the best method compared with others; one of which is the extraction of citric acid from fruits and its chemical synthesis. The production of citric acid by *Aspergillus niger* is one of the most commercially utilized examples of fungal overflow metabolism (Manil, 1967; Roukas, 1996; Ikram-ul-Haq et al., 2001). Many micro-organisms such as fungi and bacteria can produce citric acid but *A. niger* remained the organism of choice for the production of citric acid because of its genetic stability, high yields, capacity of

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using cheaper raw material (like cane- molasses) and the absence of undesirable reactions (Sikyta, 1983). According to various researches, the mutant strains might show several fold increase in citrate production as compared to wild- type cultures (Mattey and Allan, 1990; Ali et al., 2001). In this case, the exposure of this mold to the ultra violet (UV) irradiations gave mutants that exhibit high capacity of citric acid production compared to the wild parents. Within the same framework, the improveement of citric acid production can be reached by using others mutant types. For Rwanda, the nitrous acid (HNO_2) which is least expensive would be the first to be used to obtain mutant of local *A. niger*.

Cane-molasses is still the substrate of choice for citric acid production by *A. niger*. Its chemical composition varies according to the type of soil, the variety of sugar cane, the climatic conditions, the period of harvest and the employed manufacturing process (Wornick, 1969; Hendrickson et al., 1971; Curtin, 1973). In Rwanda, the Kabuye sugar refinery, in Kigali City, produces enormous quantities of molasses which would be, without any doubt, chemically different from the molasses of other parts of the world. The local molasses are rich in carbohydrates (88%) whereas the minerals are in the state of traces (A. Sibomana, National University of Rwanda, unpublished observations, 1982). The industries of Rwanda and those of other East African Community member countries spend lots of money for the importation of citric acid. However, they have at their disposal the huge quantities of molasses which are not valorized yet. Moreover, these industrial wastes are often not well-preserved and might be harmful to the environment. Thus, our work consists of the search for a strain of *A. niger* which is very efficient for the bioconversion of those deposits of local molasses to citric acid in order to insure the self-supply of this acid to local industries, the reducetion of the dependence of our nation and the environment's protection. The method that was adopted is the use of nitrous acid to create an efficient mutant of *A. niger* for citric acid production from molasses compared with the wild type.

MATERIALS AND METHODS

Isolation of *A. niger*

The used *A. niger* was isolated from the leaves of *Citrus lemon* (lemon tree) of Kigufi garden at Gisenyi. The collected leaves were packed and brought to the lab of microbiology of high institute of agriculture and animal husbandry (ISAE) at Busogo. They were cleaned with distilled and sterile water; they were cut into slides of 1 cm in diameter. The slides were put on recommended sterile and solidified culture medium (Potato Dextrose agar or PDA) contained in Petri dishes (Larpent, 1990). The cultures were incubated at 30°C for 5 days. Different colonies that appeared were biochemically and morphologically characterized. According to the proper characteristics of these microorganisms, the colonies of *A. niger* were spotted and isolated from others.

Mutagenesis of *A. niger* and mutant selection

The mutagenesis followed the protocol described by Scriban (1988). Spores of *A. niger* were put in a test tube that contained 100 ml of acetate buffer (pH = 4.0) . Thereafter, 0.15 ml of sodium nitrite ($0.2\ gl^{-1}$) was added and the mixture was incubated in bain-marie at 30°C for 1 h. To stop the reaction, 200 ml of phosphate buffer (pH = 7.0) was added to the content of the tube placed in melting ice. At pH lower than 6.0, all nitrites formed the nitrous acid which is a strong chemical mutagen that was wanted. The spores were taken out of the solution and cultivated on PDA culture med-ium at the rate of 100 l per Petri dish. The cultures were incubated for 30°C until the mushroom was matured. From the morphological and physiological characteristics of colonies, the mutant was localized, isolated and amplified.

Molasses fermentation and assay molasses

Pre-treatment of substrate

The cane molasses obtained from Kabuye sugar refinery was used in the present study. Before its fermentation, it was pre-treated according to the method of Leopold and Valtr (1969) and Prescott et al. (1981) to reduce its opacity. Depending on the volume of used flasks, 200 g of molasses were diluted in 180 ml of distilled water. To reduce metal traces whose great concentrations may bother the citric acid production, 1.88 g of potassium ferrocyanide ($K_4Fe(CN)_6$) was added. Thereafter, 10 ml of formic acid were added to the mixture and the pH was adjusted to 6 with Sodium hydroxide ($1\ gl^{-1}$). To inhibit the proliferation of other micro-organisms, the mixture was sterilized at 121°C for 15 min.

Inoculation of substrate and sampling

The cellular suspensions for the mutant and wild type of *A. niger* were prepared with an absolute asepsis. Spectrophotometrically, the cells concentrations for these two stains of *A. niger* and sugar content were obtained to be 3.10^8 cells/ml and $245\ gl^{-1}$ respectively. Thereafter, a quantity of each cellular suspension was transferred to the flasks that contained the pre-treated substrate at a rate of 50 ml by vat (400 ml of substrate). The fermentation was carried at 30°C under an agitation of 100 rpm. For the analysis, every 24 h, 20 ml were taken from every vat and were put in a separate flask. Then 1 ml of each sample was taken and put in a test tube and 9 ml of distilled and sterile water were added. The mixture was cen-trifuged at 5000 rpm for 5 min. The supernatant was salvaged and analyzed.

Measurement of sugar content

In test tubes, 200 l of distilled water, 200 l of phenolic solution (5%) and 1 ml of sulfuric acid were mixed. The mixture was homogenized in bain-marie at 90°C for 5 min in the dark and the sugar concentration was estimated spectrophotometrically at a wavelength ( ) of 480 nm.

Measurement of citric acid content

After centrifugation, the supernatant was clarified before it was

Figure 1. The obtained colonies after individualization of different mushroom, white mushroom (A), granular colonies of mature black mold (B). The colonies of black mushroom are granular generally some millimeters apart.

Figure 2. Compared colonies of wild type (SS) and mutant (SM) of *A. niger*: The colonies of mutant were very small and very close together and undistinguished by eyes, the colonies of wild type were distinct.

titrated. Using a graduated pipette, 1 ml of supernatants was diluted in 49 ml of distilled water to reinforce the discoloration and 5 drops of phenolphthalein were used to serve as color indicator. The citric acid was titrated by addition of sodium hydroxide ($1gl^{-1}$) in drops under a more or less vigorous agitation.

RESULTS

Isolation of *A. niger*

After 5 days of incubation, the whole surface of culture media was invaded by a mixture of whitish and blackish colonies of mycelia. Individualized and cultivated separately, the black mushroom formed the granular colonies with black spores whereas the mycelia of white mushroom still remained on the surface of the culture media (Figure 1). The reverse of culture medium holding the black mushroom was dominated by a sharp yellow pigment whereas it was colourless for the white mushroom. Under optical microscope, we saw that the cells of black colonies had visible spectra while these were absent in cells of white mushroom. According to their specific characteristics, the black colonies with spectra were *A. niger*.

Mutagenesis and mutant selection

In the Petri dishes that contained the mold that had been submitted for mutagenesis, after six days, we observed the tufts of blackish mold (Figure 2). Almost all the total surface of culture media was invaded by colonies of mutant strain (MS). Mutant strains were small colonies compared to the wild strain (WS). Only the magnifying glass could distinguish these colonies. Moreover, the molds which underwent the mutagenesis were short and their growth was very slow compared to that of the wild type.

Citric acid fermentation and assay methods

During the fermentation, different measurements were done within the intervals of time which were equal. As for the colour of the substrate, no change was noticed but we noticed the formation of precipitates which increased with the time. This article shows different results from the measurement of sugars (Figure 3), pH (Figure 4) of medium and citric acid (Figures 5 and 6).

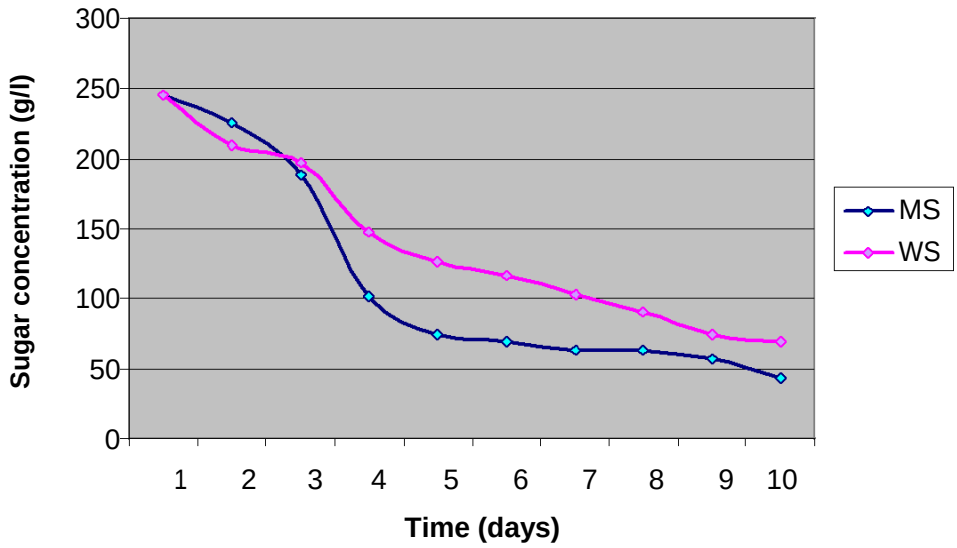

Figure 3. Variation of carbohydrates concentration in the fermentation media, sugar consumption in mutant was greater than the wild type after 2 days of fermentation.

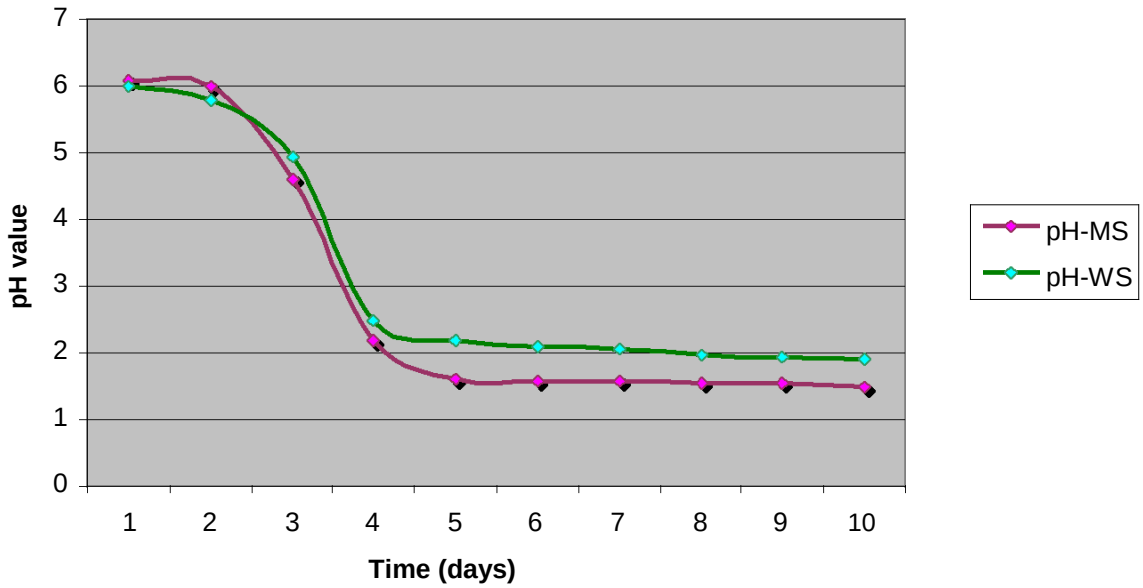

Figure 4. pH evolution within the media of fermentation. The pH diminished with the augmentation of citric acid. This diminution was due to the accumulation of this acid in the fermentation medium.

DISCUSSION

The mushrooms description given by Cooke and Whipps (1993) helped in the identification of so many types of mushrooms. In this case, the description helped to identify *A. niger* that was looked for. The black mushroom forming granular colonies and having spectra was surely the mold *A. niger* as described by this author. The white mushroom was thus another type of mushroom, that we were not interested in, which cohabited with *A. niger* on lemon tree. The isolated *A. niger* submitted for mutagenesis formed the colonies with morphological and physiological aspects which differed from those of the mother colonies. These differences testify the change in genotype of *A. niger* due to the effect of nitrous acid used as mutagen. According to Higgins et al. (2002), the colonies of mutant from the nitrous acid used are small and this is because they had difficulties in metabolism in aerobiosis

Figure 5. At equivalence point during the titration of citric acid the solution turned into dark pink because of phenolphthalein used as colour indicator.

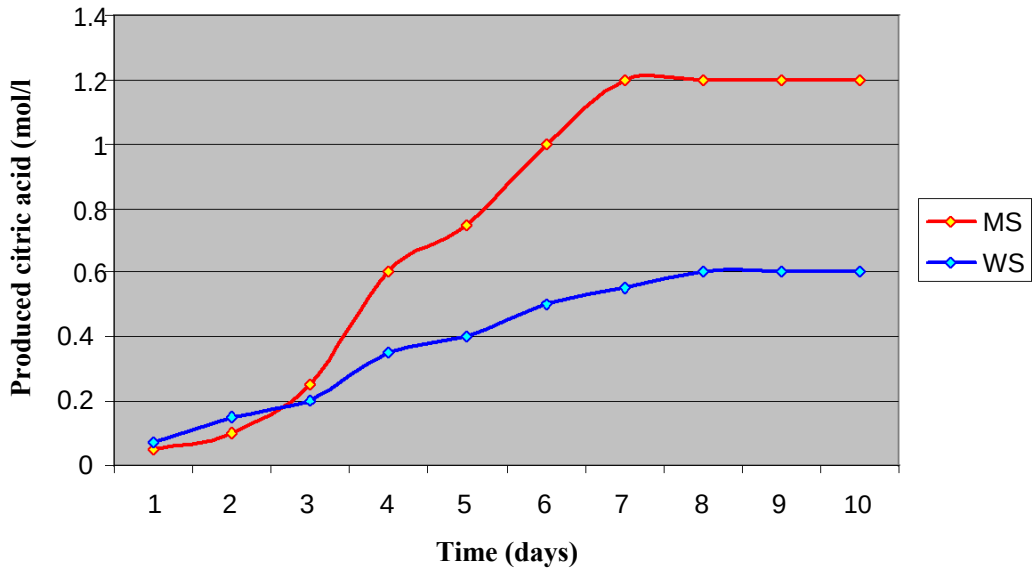

Figure 6. Evolution of the content of citric acid in fermentation media. For citric acid production, the mutant was better than the wild type (the parent).

which handicapped their growth.

As we said in the previous paragraphs, our main objecttive was the creation of mutant which exhibits high potential of bioconverting cane-molasses into citric acid. The precipitates observed during the fermentation would have been caused by the accumulation of debris resulting from digested substrate. The measurements which were done at the time of follow-up showed that the consumption of sugars contained in molasses was very intense from the 2nd day to the 5th day of fermentation.

In the two first days, the mutant showed slow sugar consumption and therefore a slow citric acid production compared with the wild parent whereas there was an opposite case in the days which followed. This situation explains the delay of adaptation among mutant type to the strange conditions which, however, are usual for the wild type. However, as soon as the mutant adapted to the conditions, it expressed all its potentials which exceed by far those of the wild strain, of converting the molasses. The continuation of sugar consumption and the diminution of pH even after the optimum time of incubation for maximal citric acid production show the occurrence of parasitic reactions after the ageing of the ferment. So, the fermentation must be stopped before the ageing of the mold to prevent those reactions.

The citric acid, in its anion form, formed the acetate; an intermediate compound of the Krebs cycle. Its consumption is due to the activity of the enzyme named aconitase (Prescott et al., 1981). To obtain the citric acid, the medium must contain the iron which inhibits the activity of aconitase and thus allows the release of acetate in extracellular medium (Daryl et al., 2003; Manil et al., 1967). In this case, the production of citric acid was due to the inhibition of aconitase by the iron from the potassium ferrocyanide added to the substrate. The amount of this acid was high in vat inoculated with the mutant comparatively to the vat inoculated with the wild type. According to the obtained quantity of citric acid, the mutant exhibited a great capacity, about two- fold increase of the conversion of the sugars contained in the molasses into citric acid more than the wild strain.

According to the literature, the nitrous acid causes genetic changes in microorganisms (Frankel et al., 1980). As a result, the proteins that result from the expression of this mutated gene will be obviously defective. An effect of this mutagen is remarkable if it touches the driving gene of any metabolism. In this case, the gene coding for PFK-2 (Phosphofructokinase-2) was the main control system of gycolysis, a crucial step of citric acid fermentation (Kornberg et al., 1970; Breitenbach-Schmitt et al., 1983; Hardewig et al., 1991). The modulation of the activity of this enzyme would be then one of the means of controlling the total flow of fermentation through the acceleration or deceleration of its steps by allowing the upstream reactions to take place permanently. This might be the case in this work. The nitrous acid would have caused the mutation that irreversibly prevented, one way or another, any kind of inhibition of Phosphofructokinase-2 (PFK-2), which is the control system of glycolysis, to stimulate the progress without disturbance of all the upstream reactions. Then, the result of this stimulation of glycolysis was the production of the citrate in a great quantity through the permanent and protected progress of this metabolism because it escaped the natural regulation that depended on the quantity of fermentable sugar in the culture medium.

Like other researchers (Mattey and Allan, 1990; Al- Zuber et al., 1991; Ali et al., 2001), the mutant of *A. niger* obtained by the use of nitrous acid as mutagen showed a great capacity of citric acid production compared to the wild type. The temperature of 30°C played a great role in optimizing the production of this acid. The mutant that we produced has got a double beneficial aspect. It is able to conduct the fermentation in usual conditions and in using usual substrate. It is endowed with a great capacity of highly consumption of the carbohydrates of substrate compared with the wild type.

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