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Improvement of *Aspergillus niger* for Sodium Gluconate Synthesis by UV Mutation Method

RAJAGOPALAN PRABU[†a,b], THOMAS CHAND[b], AND SUNHARE RAKSHA[c]

[a]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[b]Department of Bioscience, Christian Eminent Academy, Indore 452001, India
[c]Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
bioprabhu21@gmail.com

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Abstract: The sodium gluconate synthesis pathway was improved in *Aspergillus niger* by random mutation method. *A. niger* was mutated with Ultraviolet (UV) radiation and the alteration of cell bound enzymes activity of gluconic acid synthesis pathway and sodium gluconate synthesis were evaluated. The improved mutants (*A. niger* UV-112) was capable of producing sodium gluconate up to final concentrations of 60 g/L in batch fermentation, which was 3.0 fold higher than the parent strain. It have been observed that the changes in activities of cell bound enzymes related for gluconic acid pathway such as glucose dehydrogenase and glucose oxidase and it were significantly higher than the parent strain. The mutant *A. niger* strain and the simple method used to decrease the production cost and development of fermentation process for industrial production of gluconic acid or its salt.

Key words: *Aspergillus niger*, Ultraviolet (UV) radiation, Mutation, Sodium gluconate

Introduction

There are various electrochemical, enzymatic oxidation and biological method used to produce multi potential gluconic acid at industries[1]. Among the chemical conversion, such as submerged fermentation by filamentous fungi (*A. niger*) was found to be more economical methods for the production of gluconic acid in bio-industries[2]. Sodium gluconate, sodium salt of gluconic acid, are produced during the fermentation by the addition of sodium hydroxide for pH control[3]. Sodium gluconate and gluconic acid have multi potential and it were used as bottle washing agent in food industry, food additives, as well as a retardant in concrete industry[4]. The gluconic acid or sodium gluconate, commercialization has not been successful yet due to the high production cost and the low yield in dehydration process[1]. Improvement of industrial strains by recombination and random mutation methods is one of the important approaches for the development and improvement of industrial fermentation process[5]. Nevertheless, the possibleness of using

recombination or random mutation methods for the improvement of gluconic acid producing strains has not been intensively studied.

Various random mutation methods were applied for improvement of potential metabolite production lipase [6], xylitol[7], L-DOPA [8] and kojic acid [9] by microorganism has been reported. The present study was helped to improve the ability of synthesis gluconic acid enzymes activities and sodium gluconate production in *A. niger* by random UV mutational method. The gluconic acid relevant enzymes activities and fermentation process for sodium gluconate production by improved strain were carried out in shake flask level.

Material and Methods

Microorganism and media

A.niger RP-11 was isolated from Onion (*Allium cepa*) by monospore isolation method[9], and it was transferred into potato dextrose agar (PDA) slant for 4 days at 30°C for the production of spores which was subsequently used as inoculums for the fermentation. The following medium composition (g/L) Glucose 120; yeast extract 40; KH_2PO_4 1.0; K_2HPO_4 1.0; $MgSO_4$ 0.5; NaCl 1.5 Na SO_4 1.5, at pH 5.5 was used in production of mycelia and sodium gluconate.

Mutation and Isolation of high-level sodium gluconate producing mutants

The highest amount of sodium gluconate producing monospore (0.8 g /g $_{biomass}$) was used for UV mutation. In this study, Ultraviolet (UV) radiation was used as mutation agents. About 5 mL of spore solution (1 x 10^6 spores/mL) in 20 mL universal bottle was exposed to UV radiation for different periods of time (10-60 minutes). After mutation, the spore suspension was covered with dark paper and incubates 24 hours in a dark room.

The potential mutant of high sodium gluconate producing strain was isolated from the mutated spore suspension by monospore isolation method[8]. The mutated spores were washed three times with 0.1 M phosphate buffer (pH 7.0) and then diluted with 0.2 M phosphate buffer (pH 7.0) by serial dilution methods. The diluted spores were sub-cultured into PDA plate for the production of various single mutants. The isolated single colonies were selected and further sub-cultured into multiple solid media plates with above conditions.

Fermentation and Extraction of enzymes from mycelia cells

Initially, the comparisons of sodium gluconate production in mutants and non mutants strain were carried out by 12 well plate fermentation methods (1 x 10^6 spores/mL; 30°C; 240 hours). Subsequently, the selected mutant was tested using batch fermentation in 500 mL shake flask containing 150 mL medium (30°C; 200 rev/min).

The various time intervals of fermentation broths were collected during the production phase and incubated on 4°C for an hour. Subsequently, broths were filtered through vacuum filtration method and washed 2-3 times with equal portions of the phosphate buffer (pH 7, 0.01 M). About 1:1 ratio mycelia and ice chilled phosphate buffer (pH 7.2, 0.1 M) were mixed together before processed to ultrasonication[10]. The homogenate of disrupted cells were centrifuged at 4,000 × g at 4^{o}C for 15 minutes. The clear supernatant was used for enzymes assay.

Analytical Methods

The supernatant was used for sodium gluconate and glucose determination, whereas the cell concentration was determine by pellet. The concentration of sodium gluconate was analyzed using an HPLC method[1] and glucose concentration was determined by dinitrosalicylic acid

(DNS) method[11]. Glucose dehydrogenase activity was estimated according to the method as described by Lamble *et al.*,[12] and glucose oxidase enzyme activity assayed according to the Bergmeyer method[13].The cell dry weight calculation followed the Prabu *et al* method[14].

Results and Discussions

Isolation of mutated Aspergillus niger strain

The 12 well plate fermentation methods were used to isolate the mutant colonies. After 240Hrs incubation the performance of mutation were analyzed by estimating the amount of sodium of sodium gluconate. The selected mutant were further used for fermentation using shake flask method. After Mutation, the cell viability was reduced to 0.00005%, from non mutated strain. Different monospores produced various ranges of sodium gluconate. The highest sodium gluconate producer was named as *A. niger* UV-112-and poor sodium gluconate producing isolated mutants was designated as *A. niger* UV-08.

Sodium gluconate fermentation by the parent and mutated strain

The sodium gluconate production profile in batch fermentation by the parent strain (*A.niger* RP-11) is shown in **Figure 1.** The results revealed sodium gluconate synthesis has been increased until the growth stage of mycelia, where glucose was directly converted to gluconic acid and it salts. Subsequently, glucose concentration was consequently decreased in the culture which indicates that glucose was converted into gluconic acid during a growth phase. The maximum sodium gluconate was obtained (20 g/L) in between 380-420 hours of fermentation. Later, there was no increment in the amount of sodium gluconate observed; even the glucose was present in the broth during fermentation. This result brought out that the non growing cell did not have ability to convert glucose into gluconic acid.

The sodium gluconate production profiles of positive mutant, *A. niger* UV-112 (**Figure 1.**) shows that the non growing mutated cell have not capability to consume the glucose for the production of sodium gluconate in fermentation. The growing cell was only produced the sodium gluconate and maximum 60 g/L sodium gluconate was produced after about 380-400 hours. During the experiment biomass concentrations of mutated and non mutated strain were remaining constant. Moreover, the poor sodium gluconate producing strain *(A. niger* UV-08) produced maximum (7 g/L) sodium gluconate after consumed 60% of total glucose in fermentation (data not shown).

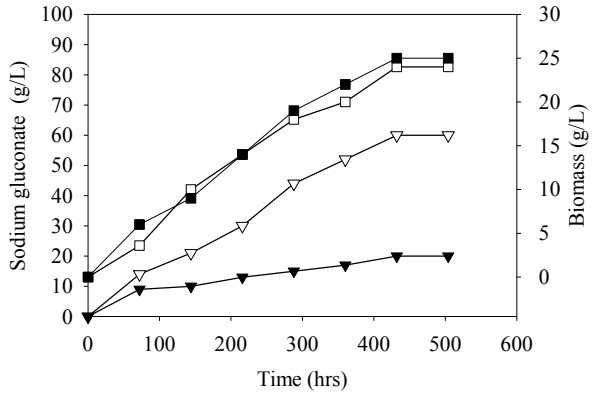

Figure 1. A typical time course of sodium gluconate fermentation. Symbols represent: (Δ) Sodium gluconate from *A.niger* UV-112; (▼) Sodium gluconate from *A.niger* RP-11; (□) cell concentration of *A.niger* UV-112 and (■) cell concentration of *A.niger* RP-11.

This above results revealed that random mutation method, using UV radiation, can be used to improve the ability of *A. niger* RP-11 for gluconic acid or salt synthesis. Normally, UV rays induce a relatively wide spectrum of mutations such as the G: C → A: T transition (prevalent), and A:T → G:C transitions (rare event)[9]. This various type of mutation has influenced gluconic acid producing fungus (*A. niger* RP-11). This discredited genome may alter the gluconic acid transcription and translation level that may increase the enzyme production or activated more active site for the binding of substrate for the conversion to the target metabolite[15].

Profile of enzymes relevant to sodium gluconate synthesis

The random mutated strains have showing highest activities of two intracellular enzymes, glucose dehydrogenase and glucose oxidase, which were directly related to gluconic acid synthesis (Figure 2). The maximum glucose dehydrogenase activity (15-17 U/mL) during sodium gluconate synthesis fermentation by the mutants was observed between 380-450 hours. This activity was approximately 2.5-2.8 fold higher than the activity detected during the fermentation by the parent strain (5.9 U/mL). On the other hand, the highest glucose oxidase activity (8.0-9.0 U/mL) analyzed on sodium gluconate produced by the mutants at 350-420 hours. Like dehydrogenase, it was detected about 2.8-3.2 folds higher than the parent strain fermentation (2.8 U/mL). Similarly, sodium gluconate synthesis efficiency of *A. niger* has been increased (Table 1) which proved that the selected mutants are capable to produce more sodium gluconate. Higher activities of both intracellular enzymes in fermentation with the mutants as compared to the parent strain were related to the higher sodium gluconate production obtained by above both strains. Moreover, negative mutant *A. niger* UV-08 was produced low amount of sodium gluconate compared with parent strain while the maximum cell concentration obtained during the fermentation. The both enzymes such as glucose dehydrogenase and glucose oxidase were depicting poor enzyme activities on during the sodium gluconate production phase.

Table 1. Performance of Sodium gluconate (SG) mutants and the parent strain

A. nigers strain	Fermentation time (H)	Maximum cell conc. (g/L)	Maximum SG conc. (g/L)	SG* yield based on glucose consumed (g/g)	Cell yield based on glucose consumed (g/g)	SG* based on cell mass (g/g)	Glucose dehydrogenase activity (U/mL)	Glucose oxidase activity (U/mL)
RP-11 (parent strain)	432	25	20	0.25	0.27	0.8	5.9	2.8
UV-112 (positive mutant)	432	24	60	0.66	0.3	2.5	17	9
UV-08 (negative mutant)	456	25	12	0.12	0.25	0.5	2.3	1.1

(*Sodium gluconate).\

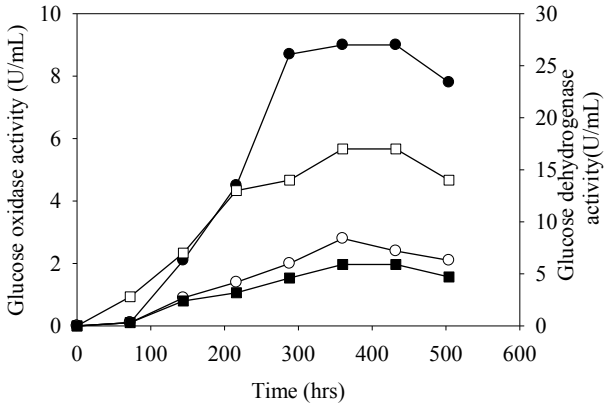

Figure 2. A typical time course of enzyme synthesis on during the fermentation. Symbols represent: (●) glucose oxidase from *A.niger* UV-112; (○) glucose oxidase from *A.niger* RP-11; (□) glucose dehydrogenase *A.niger* UV-112 and (■) glucose dehydrogenase of *A.niger* RP-11.

Among the various enzymes, glucose dehydrogenase and glucose oxidase were played important role in gluconic acid synthesis with their higher activities in the positive mutants as compared to the negative and parent strain. Probably, the mutation caused deletes the suppressor gene (frame shift) of enzymes relevant to gluconic acid synthesis and changing the conformation of the suppressor protein involved in enzymes regulations (some base substitutions). The inactive suppressor proteins could not regulate the enzymes feedback regulation for gluconic acid synthesis, which resulted to higher enzymes secretion. In order to elaborate this, further study about the signal proteins and suppressor genes for the enzymes relevant to the enhancement of gluconic acid in mutated *A. niger* need to be carried out.

Conclusion

The above UV random induced mutation studies and predicated results have been successfully used to improve sodium gluconate producing fungus, *A. niger*. The improved mutant (*A. niger* UV-112) was capable of producing higher sodium gluconate and gluconic acid synthesis enzymes activity in the mutated strains as compared to other mutated and parent strain. This simple and fast strain development method could be useful to development of fermentation process for other industrial production.

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