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Gluconic Acid Production from Golden Syrup by *Aspergillus niger* Strain Using Semiautomatic Stirred-Tank Fermenter

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Abstract

The gluconic acid batch fermentation was conducted using mutant *Aspergillus niger* NCIM 530 strain under submerged condition in 50 L semiautomatic stirred-tank fermenter. Certain cost-effective source as golden syrup was effectively utilized instead of glucose for successful industrial fermentation process. The significant level of gluconic acid (85.2 gL^{-1}) production was observed with maximum 86.97% glucose conversion over 44 hours. This process provides great advantages over traditional submerged fermentation strategies and substrates, as showed by effective production of gluconic acid by utilizing novel substrate as a golden syrup. To reduce analysis time with better accuracy, an effort has been made to use a method for evaluation of parameters like conversion of substrate and production of gluconic acid during the fermentation by using High Performance Thin Layer Chromatography (HPTLC).

Keywords: *Aspergillus niger*; Golden syrup; Batch fermentation; Gluconic acid

Introduction

Gluconic Acid (GA) is a multifunctional carbonic acid belonging to bulk chemical, with outstanding properties, including extremely low toxicity, very low corrosiveness, and a capability of forming water soluble complexes with different metal ions. Due to these physiological and chemical properties, GA itself and its salts have found extensive demand in construction, chemicals, pharmaceuticals, food, beverage, textile, leather and other industries [1-3]. There are various approaches available for the production of gluconic acid namely, chemical, electrochemical, biochemical, bioelectrochemical, and photocatalytic approach [4-7]. Because of some limitation regarding these approaches, fermentation has been proved as efficient and dominant techniques for manufacturing GA. Most studied and widely used GA fermentation process involves filamentous fungus *A. niger*. Beside *A. niger,* other species such as *A. terreus, Penicillium, Gliocadium, Scopulariopsis* and *Gonatabotrys* have been tested and reviewed [8-10]. Several bacterial species including *G. oxydans, G. diazotrophicus, Z. mobalis, A. methanolicus, P. florescens,* and the species of *Morexella, Tetracoccus, Pullularia, Micrococcus, Enterobacter and Scopulariopsis* participate in GA production [9-11].

About 50,000-60,000 tons of GA is annually produced worldwide using glucose. However, use of GA and its derivatives is currently restricted because of high prices: about US$ 1.20–8.50/kg. Refined glucose and sucrose have been the main substrate for gluconic acid production [12,13]. The process could be further economized by replacing conventional refined carbohydrate material with more economical substrates. A large quantity of raw material produced in agriculture and industrial process can be utilized for gluconic acid production to minimize the fermentation cost. The various alternative carbohydrate sources were tested to make the GA fermentation process more economical as hydrol, corn starch, can molasses, grape must, banana must, food processing residues, figs, cheese whey, beet molasses, and saccharified solution of waste paper [14].

Golden syrup is a thick, amber-colour industrial by-product in form of inverted sugar syrup, made in the process of refining sugar cane juice into sugar, or by treatment of a sugar solution with acid. It contains nearly 47% glucose; so it can be effectively utilized for gluconic acid production. In present study the process of production of GA was economized by evaluating cheapest and optimum productive substrate golden syrup using isolated *Aspergillus niger* and demonstrates its role as reservoir in biotransformation of glucose in form of golden syrup to gluconic acid in submerged fermentation conditions.

Material and Methods

Micro-organism

In present work, mutant *Aspergillus niger* NCIM 530 derived from wild type *Aspergillus niger* NCIM 530 was provided by NCL Pune, India. During the study, this strain was maintained on agar slant of molasses medium at 30°C for 4 days.

Harvesting of *A. niger* spores and inoculum preparation

The spore of mutant *A. niger* from slant were harvested in 5 ml of sterile 50 mM sodium phosphate buffer (pH 6.8) containing 0.1% pre-sterilized Tween 80. The spores in inoculums were maintained at 10^8 -10^{10} spores per ml. It was inoculated in erlenmeyer flasks containing spore germination medium with following composition: glucose 5%, di-ammonium phosphate 0.2%, $MgSO_4$, 0.25%, KH_2PO_4 0.1% (pH 5.5). The inoculated medium was put on orbital shaking incubator at 28°C with 180 rpm for 48 hrs.

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Fermenter equipment and resting cell culture system

Batch fermentations were performed in 50 L semi-automatic stirred-tank fermenter (Scigenics Equipments, India) equipped with top stirred bearing three 6 blade ruston turbine type impellers, additionally four removable baffle plates attached to shell wall of fermenter. Agitation speed of stirred was set at 250 rpm. The temperature was constantly set at point 28°C using continuous water flow with thermostat. Fermentation medium with following composition (for 100 ml of medium): golden syrup 21.28 ml (10% w/v glucose concentration), di-ammonium phosphate 0.09 gm, $MgSO_4$ 0.01 gm, Urea 0.015 gm, KH_2PO_4 0.02 gm, olive oil 2 ml, distilled water 76.72 ml (medium pH 5.5) sterilized at 12°C for 20 minutes with the help of steam air. The air saturation in fermenter was measured and controlled using polorographic dissolved oxygen probe. Before inoculation, 100% air saturation in sterilized fermenter medium was adjusted using the constant atmospheric air flow rate at 30 LPM. At same time, vessel pressure was maintained at 0.5 bars with the help of exhaust diaphragm valve. Sterilized fermentation medium was inoculated with 10% 48 hrs old inoculums medium by using peristaltic pump. The pH was measured and maintained at set point 5.5 using gel filled, glass type pH probe, by automatically adding 15% pre-sterilized $CaCO_3$ slurry. The sampling was carried out at regular interval from bottom fitted diaphragm valve.

Determination of biomass, concentration of glucose and GA

The dry fungal biomass was obtained after infiltration of broth sample though pre-weighted filter discs. The biomass was then washed with acidified distilled water, dried at 70°C and weighted [15].

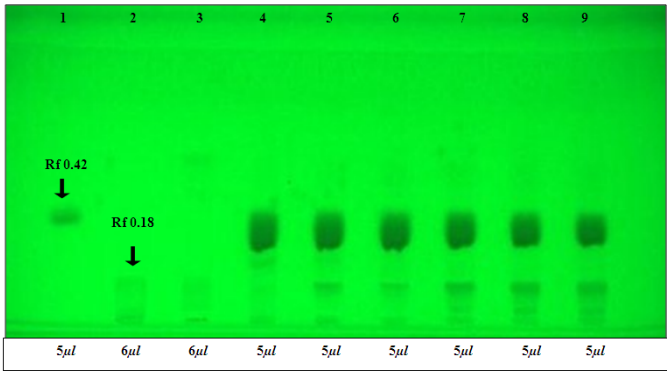
The GA concentrations in samples were determined by measuring calcium in form of calcium gluconate (CG) in fermentation broth. The glucose and GA in fermentation samples were analyzed simultaneously by HPTLC (CAMAG, Ancrom Enterprises Pvt. Ltd, Mumbai). HPTLC was performed on 10 cm × 20 cm pre-coated silica gel GF_{254} aluminum TLC plates (1.5554.0007, Merck KGaA, Germany). Plate was pre-washed with methanol: water with 4:1 (v/v) ratio and activated by heating on CAMAG TLC plate heater on 100°C for one hour. Fermented broth samples and reference (Calcium gluconate-Acros Organics Germany; Glucose-Merk) were applied to the plated by means of CAMAG (Switzerland) Linomat V TLC applicator. The plate were developed at 30°C±2°C to a distance of 8.5 cm, with 20 mL of butanol : acetic acid : water with ratio 6:2:1 (v/v) as mobile phase, in CAMAG twin trough chamber. After removal from the chamber, plate was dried on CAMG TLC plate heater III for heating for half hour at 120°C (Figure 1 and 2). Plate was scanned and quantified at 310 nm with the help of CAMAG TLC Scanne 3 with WinCATS 1.4.4 software (Figure 3 and 4). The R_f values of CG and glucose were 0.18 and 0.42 respectively.

The fermentation samples determination (Biomass, concentration of glucose and GA) were carried out in triplicate for reproducibility of results and it represented in form of mean. Statistical analysis was performed using ANOVA test software.

Results and Discussion

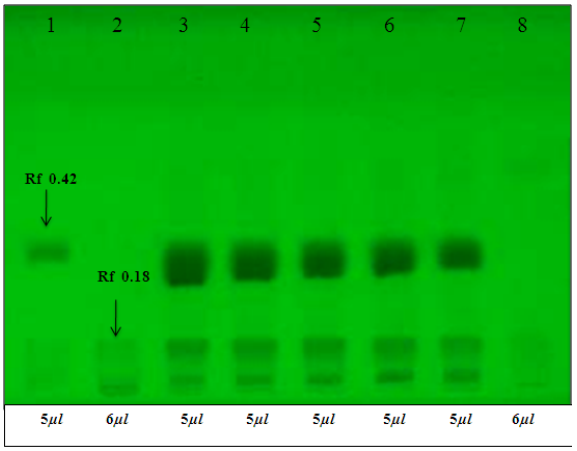
In present study, the mutant *A. niger* strain showed effective gluconic acid production with utilization of glucose in form of cheap carbohydrate source such as golden syrup (Figure 5 and 6). During batch fermentation, the initial glucose was maintained at 100 gL^{-1}. At initial residence time, GA production was increased very slowly (3.36 gL^{-1}) with optimum productivity (0.84 $gL^{-1}h^{-1}$) rate over 4 hours. An increase in the levels of GA concentration (12.33 gL^{-1}) was observed over 24 hours with 0.51 $gL^{-1}h^{-1}$ productivity and further followed by rapid increase of GA production level (34.75 gL^{-1}) after 28 hours with 1.24 $gL^{-1}h^{-1}$ productivity and 38.8% glucose conversion. So, the period between 24 hours to 28 hours is considered as peak productivity period for GA. At the end of fermentation, maximum production of GA (85.2 gL^{-1}) with highest productivity (1.94 $gL^{-1}h^{-1}$) was observed after 44 hours and showed 86.97% glucose conversion.

Several modified strategies in microbial fermentation conditions are often useful to improve product formation. Routine fermentation using naturally available microbial species under submerged conditions led to the development of the effective submerged fermentation process for GA production. This process is significantly affected by various factors. Among these, selection of suitable strain, substrate and process parameters are crucial. For significant GA production, $CaCO_3$ is used as buffering agent during fermentation [10]. However, $CaCO_3$ limits the possibility of obtaining high concentrations of product due to the



Track no 1 = 25 µg std. glucose (Merk)
Track no 2 = 180 µg std. calcium gluconate (Acros Organics, Germany)
Track no 3 = 6 µg std. gluconic acid (Acros Organics, Germany)
Track no 4 to 9 = 4, 8, 12, 16, 20, 24 hours fermentation sample

Figure 1: HPTLC chromatogram of GA fermentation samples (4-24 hours) at UV 254 nm.



Track no 1 = 25 µg std. glucose (Merk)
Track no 2 = 180 µg std. calcium gluconate (Acros Organics, Germany)
Track no 3 to 7 = 28, 32, 36, 40 & 44 hours fermentation samples
Track no 8 = 6 µg/µl std. gluconic acid (Acros Organics, Germany)

Figure 2: HPTLC chromatogram of GA fermentation samples (28-44 hours) at UV 254 nm.

limited solubility of the resulting calcium gluconate (4% at 30°C) that precipitates over the mycelium thus inhibiting oxygen uptake [16]. To overcome this problem, substrate concentration was maintained up to 10%. In the present batch fermentation, $CaCO_3$ slurry (15%) was continuously provided and maintained at constant pH (5.5) until the end of process. This was showed by increase volumetric productivity for GA production. Agitation increases the efficiency of aeration resulting in an increased interface between the gas and the liquid [17]. Because foaming affects on oxygen transfer rate from air and creates several problem in microbial aerobic fermentation, 2% olive oil was used as an antifoaming agent during fermentation [18].

Generally glucose is used as carbon source for microbial production of gluconic acid. Much research work on gluconic acid production has been done in cultures of *A. niger* using glucose as sole carbon source. For economical consideration of fermentation process, various types of alternative carbohydrate materials such as hydrol, corn starch, grape must, banana must, fig, cheese whey, food processing residues, and saccharified solution of waste paper were utilized in submerged and solid-state surface fermentation [19-24].

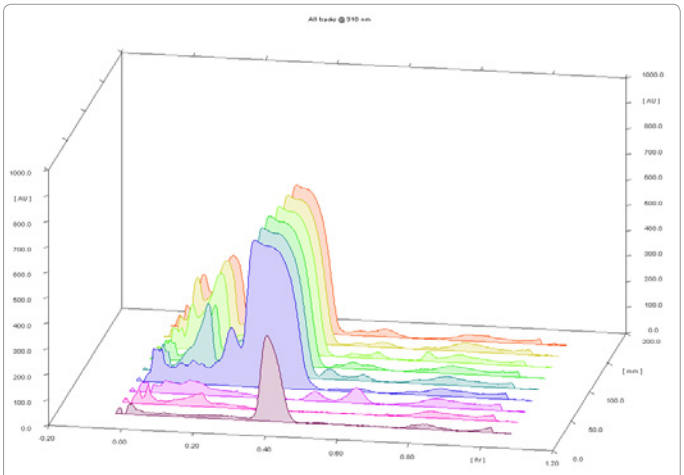


Figure 3: Densitogram curves of GA fermentation samples (4-24 hours) at 310 nm.

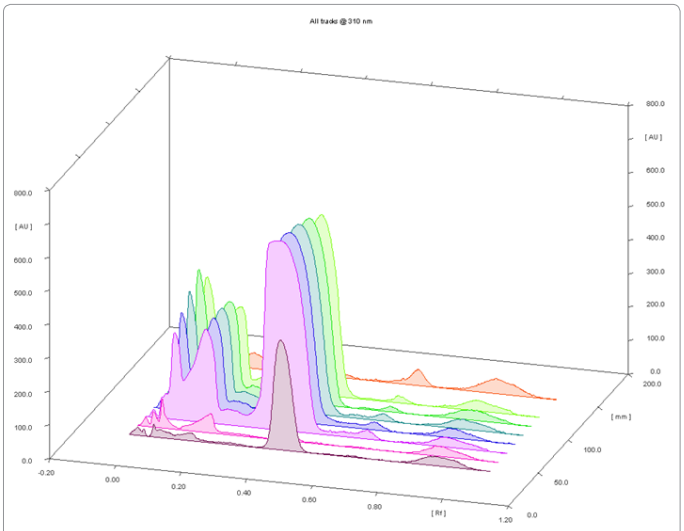


Figure 4: Densitogram curves GA fermentation samples (28-44 hours) at 310 nm.

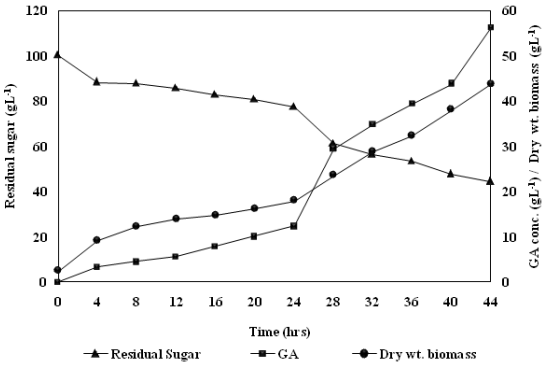


Figure 5: Time course changes of glucose, GA concentration and dry biomass weight during GA batch fermentation with *A. niger*.

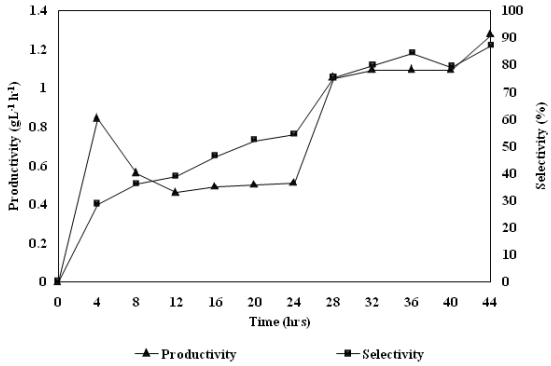


Figure 6: Time course changes of productivity, selectivity and yield during GA batch fermentation with *A. niger*.

Sing et al. [15] reported GA production by *Aspergillus niger* ORS-4.410 using rectified grape must, banana must and treated molasses must under submerged fermentation, it showed GA production 73.2 gL^{-1}, 69.3 gL^{-1} and 58.32 gL^{-1} with product formation rate 0.509, 0.481 and 0.405 respectively [14]. Sapkal and Kulkarni (2002) reported GA production in a continuous recirculation reactor using immobilized *A. niger* on cellulose microfibers and obtained 158 g/l GA with 0.28 $gL^{-1}h^{-1}$ production rate [25]. They also immobilized *A. niger* on cellulose and obtained 135 gL^{-1} of GA with 0.09 $gL^{-1}h^{-1}$ productivity rate in surface culture [26]. The scientist attempted utilization of waste paper for GA production as 80-100 gL^{-1} achieved with 0.04 $gL^{-1}h^{-1}$ productivity [14]. The kinetic analysis of gluconic acid production by *A. niger* mutant ORS-4.410 indicated that fermentation of glucose results in 94.5% yield after 144 hours of incubation [27]. This paper represents first experiment using raw golden syrup as glucose containing substrate for GA fermentation in semi automatic PLC based bioreactor using mutant *A. niger* NCIM 530 strain. It showed average yield of GA 85.20 gL^{-1} over 44 hours with 1.94 $gL^{-1}h^{-1}$ productivity. However it is apparent that pure glucose was the better carbon source resulting high yield of GA as compared to golden syrup. Because, presence of complex carbohydrate residual material (remaining unconverted starch, other glucose etc) or some impurity in golden syrup may retard mutant *A. niger* NCIM 530 to favor the GA production.

To obtain high level of GA production a sufficient level of pure oxygen supply is indispensible [28,29]. However, in present study pure form of air supply were used during fermentation process. Higher

productivity may have been obtained if a sufficient level of pure oxygen supply.

In GA fermentation process, analysis of fermented samples has importance for evaluation of microbial process including GA concentration, yield determination, productivity etc. Many researchers used HPLC or enzymatic spectrophotometric method for analysis of gluconic acid. However, these methods are costly and time consuming. We made effort to develop the HPTLC method for analysis of GA and glucose during fermentation process. This saves cost per analysis and analysis time as well. It also facilitates repeated detection (scanning) of the chromatogram with same or different parameter. It has main advantage to analyse both glucose and GA concentration of many sample (nearly 15 samples) in single step with better accuracy.

Economical feasibility is of prime importance for any industry significant product formation to be viable and successful, and to a greater extent depends on selection of substrate for fermentation process. In addition, the proper fermentation conditions are needed to improve product formation during microbial fermentation. The present work made attempt to obtain optimum GA production with minimum residence time by using novel carbohydrate sources as golden syrup in replacement of glucose during submerged fermentation, but process still needs some experimentation and improvement. It may be possible to establish effective strategies such as substrate clarification, continuous GA fermentation and simultaneous recovery of GA.

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