JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2011. Vol. **5**(1), p. 235-239

# Effect of Nutrient Additives on Gluconic Acid Production by *Aspergillus niger* during Submerged Fermentation

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(Received: 27 September 2010; accepted: 12 November 2010)

**In present study, the various supplementations of different cheaper additives were analysed for deducing their role in GA fermentation by *Aspergillus niger*. In addition of antifoaming property, soybean oil (0.5%) and olive oil (2%) efficiently increases the GA production by 11.2 $gL^{-1}$ and 5.61 $gL^{-1}$ over 36 hours respectively. These additives also showed significant contribution in biomass increment. However, the remarkable results were observed in cases of yeast extract (YE) and $CaCl_2$ supplementations in fermentation media. Among these, YE (2%) markedly increased GA production yield (96.16 $gL^{-1}$) up to 58.29% over 36 hours. The low concentration of $CaCl_2$ (0.5%) also have potential to increase the yield of GA up to 14.14%. The effect of various hydrocarbons on GA concentration in fermentation was also studied. The ethanol acts as inducer of GA production, where as methanol and hexane shows the negative effect on GA concentration during fermentation.**

**Key words:** *Aspergillus niger*, Gluconic acid fermentation, Soybean oil, Olive oil, Yeast extract, $CaCl_2$, Hydrocarbons.

Organic acid constitute a key group among the various building block chemicals that can be produced by microbial process. Among various organic acid, gluconic acid and its salts are used in different industrial application due to some outstanding properties including extremely low toxicity, very low corrosiveness and a capability of forming water soluble complexes with different metal ions. Gluconic acid is absolute polyhydroxycarboxylic acid that used as bulk chemical in beverage, food, textile, pharmaceutical, and construction industries[1].

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Because of extensive demand of gluconic acid nearly 50,000-60,000 tonnes annually worldwide, it produced commercially via microbial fermentation using glucose as major carbohydrate source. There are many reports on the fermentative production of gluconic acid and its salts by various bacterial (*G. oxydans, Z. mobilis, A. methanolicus, P. fluorescens* etc) and mold species (*A. niger, A. pullulans, Penicillium, Gliocadium*). Gluconic acid is produced from glucose through a simple dehydrogenation reaction catalyzed by glucose oxidase enzyme[2]. The favored production process is submerged fermentation by *Aspergillus niger* utilizing glucose as a major carbohydrate source, which accompanied product yield of 98%. However, use of GA and its derivatives is currently restricted because of high prices about US$ 1.20–8.50/kg[1]. So, its need to modified fermentation process for the development of an effective and economical system for GA production.

In addition of glucose substrate and inorganic constituents, the various cheaper additives may contribute their role in GA production. The addition of vegetable oils in fermentation media can enhance the rate of oxygen transfer rate in medium and accelerate rate of GA formation[3,4,5]. The various hydrocarbons also enhance GA concentration during fermentation. Li and Chen (1994) reported an increase the GA production by enhanced intracellular glucose oxidase activity by 43%, 110% and 31% on addition of n-dodecane, n-hexadecane and soybean oil, respectively.

In the present report an attempt was made to demonstrate the role of various cheaper additives (soybean oil, olive oil, YE, $CaCl_2$, ethanol, methanol and hexane) on GA production during the fermentation by *A. niger* using glucose as sole carbon source.

## MATERIAL AND METHODS

**Microorganism**

A gluconic acid producing strain *A. niger* was isolated from bagasse waste of sugarcane industries. During the study, the strain was maintained on potato dextrose agar slants at 4-6 $^0$C and subculture every month.

**Harvesting of spores and inoculum preparation**

The spore of *A. niger* from slant were harvested with the help of 0.1% pre-sterilized Tween 80. The spores in inoculum were maintained at $3 \times 10^6$ and it was inoculated into spore germination medium. During inoculum preparation, 10% of Tween (0.1%) spore suspension was used and inoculated medium (Table 1) was put on orbital shaking incubator at 28 $^0$c with 150 rpm for 48 hrs.

**Gluconic acid fermentation**

For gluconic acid production, 10 ml inoculum added in 250 ml Erlenmeyer flask containing 90 ml of GA fermentation medium (Table 1). Pre-sterilized calcium carbonate slurry (40 $gL^{-1}$) was used as neutralizing agent and separately added to medium. The flasks were incubated at 28 $^0$C on orbital shaker at 150 rpm up to 36 hrs. After incubation, analyses of samples were carried out. To investigate the influence of various additional nutrients namely soybean oil, olive oil, YE, $CaCl_2$, ethanol, methanol and hexane etc were added in varying concentration (0.5-2%) along with control.

**Analytical methods**

**Determination of dry biomass**

Culture fluid was filtered through Whatman No 1 paper. The filtered mycelia were washed with acidified (pH 2.5 with 4M HCl) distilled water. The separated mycelia were washed several times with demonized water until the pH of spent wash was 7.0. Then mycelia were dried (75 $^0$C) to a constant weight. And its dry weight was determined by subtracting the average predetermined dry weight of Whatman No 1 paper from the combined weight of Whatman No 1 paper along with mycelium[5].

**Determination of glucose and gluconic acid**

The glucose content in filtrate sample was estimated by standard Di-nitro-salicyclic acid (DNSA) method using glucose as standard[6]. The quantitative analysis of gluconic acid formed in fermentation broth was performed by measuring the concentration of Ca-gluconate in the medium indirectly by assaying the dissolved calcium amount in culture medium[7].

## RESULTS AND DISCUSSION

The balancing the nutrient components are important in setting up medium for submerged gluconic acid fermentation by *A. niger*[1]. Lower concentration of a few nutrients appeared supportive for gluconic acid production. So, varieties of additional nutrients were supplemented to the fermentation medium, the fermentation was carried out for 36 hours and results are depicted in Figs. 1-4.

**Table 1.** Media composition for inoculum medium and fermentation medium

| | Inoculum medium($gL^{-1}$) | Fermentation medium($gL^{-1}$) |
|---|---|---|
| Glucose | 50 | 100 |
| Di-ammonium phosphate | 2 | 0.9 |
| Urea | - | 0.11 |
| $MgsO_4$ | 2.5 | 0.15 |
| $KH_2PO_4$ | 1 | 0.20 |
| pH | 5.5 | 5.8 |

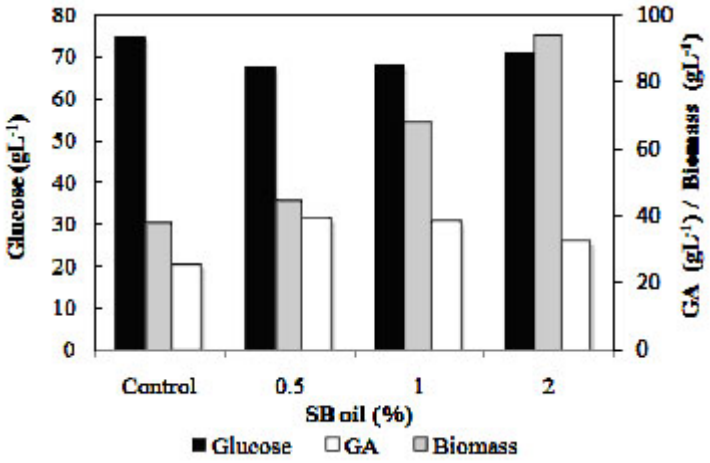


**Fig. 1.** Effect of varying concentrations of SB oil on gluconic acid production by *A. niger*

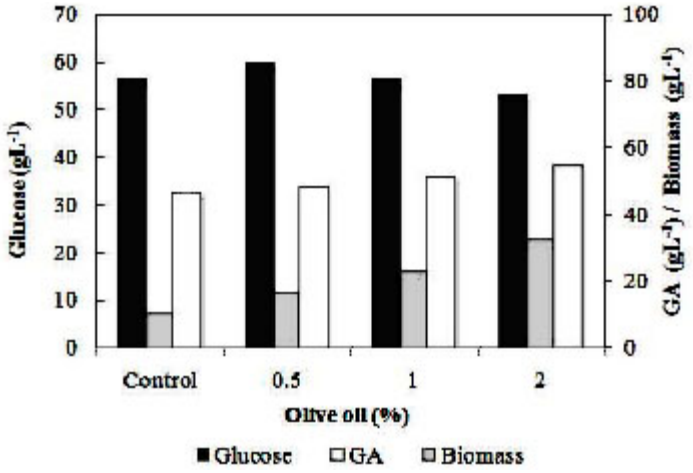


**Fig. 2.** Effect of varying concentrations of olive oil on gluconic acid production by *A. niger*

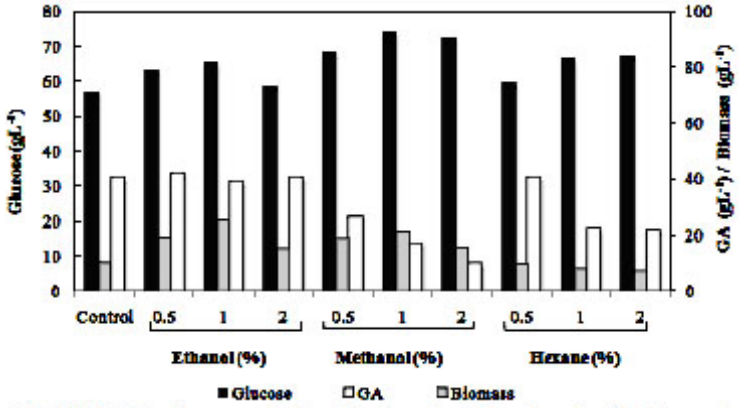


**Fig. 3.** Effect of varying concentrations of hydrocarbons (ethanol, methanol and hexane) on gluconic acid production by *A. niger*

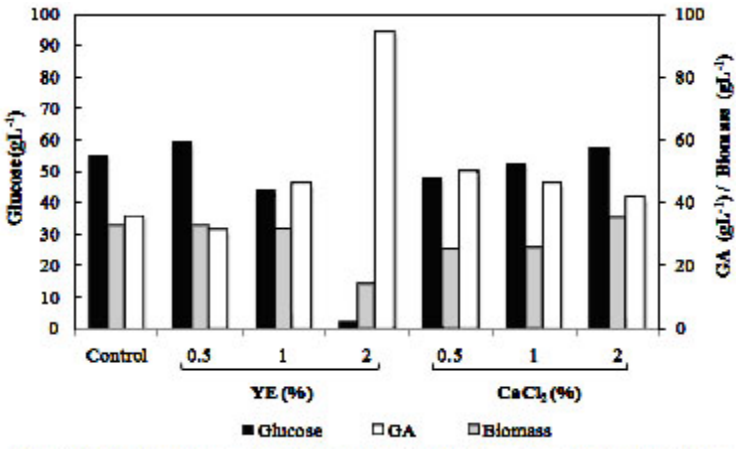


**Fig. 4.** Effect of varying concentrations of YE and $CaCl_2$ on gluconic acid production by *A. niger*

Generally oils include SB oil and olive oil used as antifoaming agent. Because foaming affect on oxygen transfer rate from air and creating several problem in microbial respiration in aerobic fermentation[8]. Enhanced level (11.2 $gL^{-1}$) of GA production and 16.87 % of increased selectivity was observed when 0.5% of SB oil was incorporated in fermentation (Fig. 1). However, SB oil with 1% & 2% showed less stimulation effect of GA production (30.83$gL^{-1}$ & 25$gL^{-1}$ respectively) as compared to control (20.18 $gL^{-1}$). The increased may be due to antifoaming property and presence of nutritive constituent in SB oil. The SB oil also showed significant contribution in biomass increment.

The GA production significantly increased with respect to olive oil (Fig. 2) with comparison to control, GA production increase by 1.13$gL^{-1}$, 3.37 $gL^{-1}$ & 5.61 $gL^{-1}$ when fermentation supplemented with olive oil (5-2%) respectively. In addition, it was observed as inducer for biomass enhancement. It was significantly increased the biomass up to 22.1 $gL^{-1}$.

Roukas (2000) reported that concentration citric acid and gluconic acid was increase in addition of 6% methanol. The effect of methanol is permeability level, allowing metabolites to be excreted from cell[9]. Glucose oxidase concentration in *A. niger* could be increased by the inclusion of various hydrocarbons during cultivation. So, in the present attempt was made to study the effect of various volatile solvent and hydrocarbons (ethanol, methanol & hexane) on gluconic acid production by *A. niger* (Fig. 3).

Due to addition of ethanol (0.5%), GA concentration was increased by 1.12 $gL^{-1}$. The GA production was declined as increasing concentration of ethanol. So, it should be provided at low concentration for GA production by *A. niger.* However, methanol and hexane showed adverse effect on GA production. The productivity was observed in decreasing form as 0.9-0.47 $gL^{-1}h^{-1}$ (hexane), 0.59-0.02 $gL^{-1}h^{-1}$ (methanol) and they also retarded biomass formation during fermentation. So, both methanol and hexane act as negative regulator for gluconic acid production activity of *A. niger.*

The yeast extract acts as good source of nitrogen and some important vitamins may assist in microbial growth[10,11]. The glucose activity of *Penicillium vitale* was enhanced up to 40% due to presence $CaCl_2$ in fermentation medium[12]. When yeast extract and $CaCl_2$ was used as additive during fermentation process, the promising results was observed (Fig. 4). In case of yeast extract (2%), the GA production was marked increased by 58.29 $gL^{-1}$ with entire consumption of glucose. It showed 94.16% selectivity and 2.62 $gL^{-1}$ $h^{-1}$ production rates. The $CaCl_2$ also showed positive results in favors of GA production. Enhanced level (14.17 $gL^{-1}$) of GA concentration, 1.40 $gL^{-1}h^{-1}$productivity & 96.30% selectivity were obtained from 0.5% of $CaCl_2$. The 2% $CaCl_2$ showed the highest selectivity up to 97.64 %.

## CONCLUSIONS

In conclusion, the present study made first effort for improvement in performance of fungal species for gluconic acid production using various cheaper additives including vegetable oils, hydrocarbons & nitrogen source etc, were employed. Among these, yeast extract was markedly increased the GA yield (96.16 $gL^{-1}$) up to 60 % with short time. Both olive oil and soya oil were contributing their role in increases GA production (11.20% and 5.61% respectively) along with the biomass formation with during submerged fermentation. The low concentration $CaCl_2$ (0.5%) also have potential to increase the yield of GA up to 14.14%. In case of ethanol, it acts as inducer of GA production by *A. niger*. However, both methanol & hexane show the negative production of GA due to their presence in fermentation. Although further experimentation is still needed to increases the GA production with shorter fermentation time and made submerged fermentation process feasible.

## ACKNOWLEDGEMENTS

The authors are grateful to the Bioprocessing & Herbal Division, Mahatma Gandhi Institute for Rural Industrialization Wardha, Maharastra, India for Scientific Research and partial support of this work.

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APPENDIX:

GA- Gluconic acid
YE- Yeast extract
SB oil- Soybean oil
GA productivity- [gm gluconic acid per liter per hour]
Selectivity (%) - [(gm gluconic acid per gm consumed glucose) x 100]
Yield (%) - [(gm gluconic acid per gm feeding glucose) x 100]