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SOLID STATE FERMENTATION OF *ASPERGILLUS NIGER FOR* CITRIC ACID PRODUCTION USING AGRICULTURE RESIDUE AS SUBSTRATE

***Irum Javed[1], Muhammad Asgher[2], Sadia Noreen[1], Humara Naz Majeed[1] and Tanzila Sahar[1].**

[1]*Departent of Biochemistry, Govt. College Women University, Faisalabad, Pakistan*

[2]*Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan*

*Corresponding Author**:** irumnaveed78@gmail.com

ABSTRACT

In recent years, the demand for citric acid production has been increased due to its application in various advanced medical fields, which can only be fulfilled by biotechnological fermentation using agro-industrial wastes. It is a bidirectional beneficial process for cost effective manufacturing of citric acid and management of agro-industrial waste products. This project was carried out in order to evaluate the enhanced production of citric acid by *Aspergillus niger* using agro-industrial wastes. In the first step of study the liquid state fermentation (LSF) and solid state fermentation (SSF) by *Aspergillus niger* was compared using molasses based medium and the different carrier substrate. In a second set of study wheat bran was utilized for citric acid production via fermentation parameters was optimized. The results showed significant ($P \leq 0.05$) difference among LSF and SSF and between different substrates in LSF and SSF. Wheat bran showed significantly increased ($P \leq 0.05$) production of citric acid ($P = 0.001$) compared to other carrier substrates used through SSF. It was observed that 4.5 pH, 60 % moisture content, 5ml/5g inoculum size and 30^{o}C temperature, are the initial requirements for maximum production of citric acid. It was also observed that Yeast extract in 1% concentration was the best nitrogen source. Surfactants like Tween 80 and sodium dodecyl sulphat (SDS) showed no effect on the citric acid.

Keywords: Agro-industrial waste, molasses, solid state fermentation, Liquid state fermentation, *Aspergillus niger*.

1. INTRODUCTION

Citric acid (2- hydroxy-1, 2, 3, propane tricarboxylic acid) is the one of the most multipurpose organic acid. It is essential constituent of various food preparations, pharmaceuticals, synthetic biodegradable detergents, cosmetics, alkyl resins and many other products (Barrington and Kim, 2008). In the last decade, it was used as acidulant, antioxidant, preservative, plasticizer synergistic agent and as flavor enhancer, but with advancement in biotechnology and medical sciences it is now demanded for its applications in biomedicine for synthesis of biopolymers to culture human cell lines, nanotechnology for drug delivery systems and agriculture as a powerful sequestering agent for bioremediation of heavy metals. The by-products as a cheap carbon source remained significantly important equally for biological detoxification of agro-industrial wastes as well as cost reduction in citric acid production (Kulkarni, 2015). Previous Studies revealed the production of various chemicals like glucose, ethanol, citric acid, starch, tartaric acid by using agro-industrial waste (Ukwuru and Egbonu, 2013), which can provide suitable environment for the growth of microorganisms due to presence of nutrients like sugars, proteins, minerals and water (Mussatto *et al*., 2012). *Aspergillus niger* one of the most common species that is used for the production of many industrial substances, preferably for citric acid (E330) and gluconic acid (E574)

(Kobomoje *et al*., 2013).

Citric acid is produced commercially, by submerged fermentation (SmF) of starch or sucrose containing media using *Aspergillus niger* (Barrington and Kim, 2008). However, the SSF has got much interest, because of higher yields than those in submerged cultures with low water content, reduced possibilities of contamination by bacteria and yeast, friendly environment for growth of fungi, higher levels of aeration, especially adequate in processes that demand an intensive oxidative metabolism, uniform dispersion of spores throughout the medium and low energetic requirements (Mussatto *et al*., 2012; nee'Nigam, 2009). Furthermore, it was also stated that physico-chemical parameters, particle sizes along with nutritional factors as nitrogen, phosphorous, potassium and other salts also influence the growth of *A. niger* and production of citric acid in solid substrate fermentation (Lotfy *et al*., 2007).

The main objectives of the present study were to compare liquid state fermentation and solid-state fermentation for the citric acid production from sugarcane molasses as a nutrient along with easily and abundantly available agro-industrial waste as inert support/substrate using *A. niger* and also to optimize different parameters for high citric acid yield and concentration.

2. MATERIALS AND METHODS

2.1 Substrates and nutrient source used for Fermentation

Molasses obtained from Crescent Sugar Mill, Faisalabad was used as a nutrient source and substrate for the production of citric acid through solid state as well as liquid state fermentation. Different agro-industrial wastes (Wheat straw, Wheat bran, Banana stalk, Baggasse, Rice husk, Corn stover) were dried at 70 °C and threshing through waley mill in institute of soil and environmental sciences, UAF obtained 40mm mash size particles. The powdered material was stored in air tight plastic jars to keep them free of moisture.

2.2 Fungal culture and inoculum preparation

Pure culture of *Aspergillus niger* was obtained from Industrial Biotechnology Lab (IBL) University of Agriculture, Faisalabad, was raised on Potato dextrose agar (PDA) slants at 28 ºC for seven days. Slants were preserved at 4 ºC for up to two months. Vogel's medium (100 ml) without agar was prepared; the pH of the medium was adjusted to 4.5 with 1 M HCl/ 1M NaOH and autoclaved for 15 min at 121°C under the pressure of 15 lb/in^2. After cooling at room temperature, the spores of *A. niger* from PDA slants were transferred into the flask aseptically in laminar air flow.

2.3 Liquid and Solid State Fermentation

2g of each wheat straw, wheat bran, banana stalk, sugarcane baggasse, rice husk and corn stover were taken in the separate duplicate conical flasks. Molasses solution (10 %) was adjusted to pH 5.5 using 1M NaOH /1M HCl and 100 ml added in each flask containing substrate (Table 3.3). Marble gravels (2-3) were also added in the flasks and the flasks were autoclaved (121°C) for 15 min under the pressure 15 lb/in^2. After sterilization 2ml inoculum was added aseptically to each flask in the laminar flow by using sterilized pipette. The flasks were incubated at 30°C for 5 days in rotary shaker operating at 120 rpm.

5g of each of the substrate were taken in separate duplicate flasks. The substrate in each flask was moistened by using molasses solution prepared in the distilled water and adjusted to pH 5.5 using 1M NaOH /1M H_2SO_4 to 60% moisture level (Table 3.4) and

autoclaved (121°C) for 15 min. After cooling 5ml inoculum was added aseptically in each flask under the laminar flow with the help of sterilized pipette. The flask was incubated at 30°C for 3 days under still culture conditions.

2.4 Citric acid extraction

After the completion of stipulated fermentation samples were harvested for citric acid estimation. In case of liquid state fermentation, the fermented samples were filtered through wattman No. 1 filter paper and the filtrate was used for citric acid analysis. In case of solid-state fermentation, 100 ml distilled water was added to the fermented flask and the flasks were shaken (150 rpm) for half an hour. The contents were filtered by using wattman No. 1 paper and the filtrate was centrifuged at 1000 rpm for 10 min and supernatant was used for citric acid analysis. Citric acid was analysed in a mixture containing 1 ml of culture supernatant, 1.30 ml pyridine and 5.7 ml of acetic anhydride was added into test tubes. The absorbance was measured at 405 nm on a spectrophotometer (T-60, PG Instruments, UK). Standard factor was obtained by dividing concentration/absorbance and mean standard factor was calculated. Regression equation was fitted statistically and standard curve was obtained.

***2.5* Optimization of parameters**

To find out the most suitable pH, moisture content, inoculum size, temperature, nitrogen constituent (ammonium nitrate, ammonium sulphate, urea, peptone, Yeast Extract) and effect of surfactant (tween 80, sodium dodecyl sulphate) were evaluated for maximum citric acid production.

2.6 Statistical analysis

The data obtained were analyzed by ANOVA to determine the significance of results using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Mean ± SD was calculated using Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1 Comparison of Liquid and solid state fermentation and selection of substrate for optimization of parameters

The present work constitutes an attempt to design optimization strategies for the citric acid production by *A. niger*. In the primitive studies citric acid production by *A. niger* was compared in solid state and liquid state fermentation by using 10% molasses solution in LSF and SSF. The results revealed a significant ($P \leq 0.05$) difference among LSF and SSF and between different substrates in LSF and SSF. It was observed that SSF is more efficient than LSF for citric acid production. Wheat bran showed significantly increased ($P \leq 0.05$) production of citric acid ($P = 0.001$) (Figure 1). In SSF wheat bran showed 12.65±0.100 mg/ml citric acid yield as compared to 12.05±0.160, 10.56±0.330, 11.44±0.230, 11.46±0.560, 12.05±0.160 mg/ml with, wheat straw, banana stalk, bagasse, rice husk and corn stover, respectively (Figure 2). It was also observed that SSF yield high weight of biomass as compared to the LSF. Kumar *et al*. and Prado *et al*. reported that Solid State Fermentation (SSF) has the potential to increase the efficiency of citric acid production and in the recent years there has been increasing interest in the use of SSF process as an alternative to submerged fermentation (Kumar *et al*., 2003a; Prado *et al*., 2004)

3.2 Effect of pH on citric acid production

The results showed significant ($P \leq 0.05$) difference with respect to the citric acid production at different pH ($P = 0.049$). the pH 4.5 found best for the molasses medium containing wheat bran for the citric acid of production (15.335 mg/ml). However, cell biomass

was high at pH 6.5 (2.845 g). At the pH 4.5 there was less production of biomass (2.520 g). By further increasing the pH there was lower production of citric acid and increased production of biomass (Figure 3.2).

These findings were similar to those of Kim *et al.* who reported that metabolic activity of fungus is very sensitive to pH level of media. The initial pH of the solid substrate showed an effect on citric acid production by A. niger (*Kim et al.,* 2006) The initial pH from 2 to 6 is often used in solid substrate and submerged fermentation for the citric acid production by *A. niger* (Adham, 2002; Lesniak *et al*., 2002). Kristiansen observed that *Aspergillus foetidus* produced a maximum concentration of (6 g/l) of citric acid at pH 3.5. (Kristiansen and Sinclair, 1979).Owing to genetic variability various researchers have reported various pH optimal values for different culture strains. Femi-Ola and Atere also observed that the maximum citric acid was produced at 4.5 pH value and no significant difference in the biomass weight at different pH ($P \geq 0.05$), while significant differences were observed ($P \leq 0.05$) in the amount of citric acid produced and biomass production during fermentation (Femi-Ola and Atere, 2013).

3.3 Effect of moisture content on citric acid production

Results showed significant ($P \leq 0.05$) difference with respect to the citric acid production at different moisture levels ($P = 0.001$). The wheat bran at pH 4.5 for 60% (w/v) was the best mioisture level for the citric acid production. At the moisture level of 34% (w/v) there was higher production of biomass (2.805 g). As the moisture level increased beyond 60% the citric acid as well as biomass production decreased (Figure 3).

The initial moisture content has impacts on the solubility of nutrient, heat and gas transfers and swelling of substrate (Ellaiah *et al*., 2004). If the moisture content of the substrate is too low, it causes reduced citric acid production due to low nutrient solubility and poor substrate swelling (Nampoothiri *et al*., 2004) If the moisture content increases more than the substrate's retention capacity, it causes insufficient aeration and reduced mycelium growth (Battaglino *et al*., 1991). Kumar *et al.* reported for citric acid production by A. niger on fruit waste, range of moisture content from 65-85% was found best (Kumar *et al*., 2003b). Roukas and Mahadik *et al.* found that moisture content ranging from 70 to 80% increased citric acid and lipase production by *A. niger* using semi-dried figs and wheat bra (Mahadik *et al*., 2002; Roukas, 2000). In present study 60% moisture content gave the best results. It may be due to the difference of *A. niger* strain and composition of growth medium.

3.4 Effect of Inoculum level on citric acid production

The results revealed significant ($P \leq 0.05$) difference with respect to the citric acid production with different inoculum levels ($P = 000$). The 5ml inoculums gave the maximum citric acid yield. It was observed that the flask containing 2ml inoculum produced less biomass in SSF (2.495g) whereas there was increased production of biomass (2.860g) in the flask containing 6ml inoculums (Figure 4).

Uyar and Baysal reported that high inoculum concentration causes over- crowding population, nutrient competition and rapid utilization of nutrients (Uyar and Baysal, 2004) The low inoculum concentrations, decreases metabolite production,

while increases the risk of contamination due to an insufficient cell population. According to the previous studies, an inoculum concentration between 1 x 10^4 to 1 x 10^9 spores/ml was observed to be suitable for citric acid production by *A. niger* (Adham, 2002; Ruijter *et al*., 2000).

3.5 Effect of temperature on citric acid production

The results revealed significant (P< 0.05) difference with respect to the citric acid production at different incubation temperatures (P= 0.005). It was observed that 30°C was the best temperatures for the maximum (17.15 mg/ml) production of citric acid. By increasing the temperature there was decrease in citric acid production. By increasing the temperature from 25°C to 35°C there was increase in biomass production from 2.280g to 2.68 g but at the temperature 40°C there was slight decrease in biomass *i.e.* 2.13g. (Figure 5). Temperature above 30ºC was to be inhibitory for citric acid yield as it causes inhibit culture development and increased byproducts.

Higher than optimal temperatures result in denaturation and inhibition of enzyme, extra moisture loss and growth arrest while lower temperatures lead to lower metabolic activity (Adinarayana *et al*., 2003). Selahzadeh and Roehr reported that the temperature of the fermentation media affects yields as well as rates of citric acid fermentations (Selahzadeh and Roehr, 2003). The result of this study was in line with Narayanamurthy *et al*. who worked on areca husk for citric acid production by *Aspergillus niger* through SSF (Narayanamurthy *et al*., 2008). Ikram-ul-Haq *et al*., (2002) reported that most filamentous fungi are mesophilic requiring optimal temperatures between 25-35°C, some species thrive at 50°C (Ali *et al*., 2002). Ali *et al*. (2016) observed maximum citric acid yield at 30 °C in batch culture and increase in temperature suppressed the citric acid yield potential of the culture used (Ali *et al*., 2016). The results thus give emphasis to the economic significance of suitable temperature control in industrial citric acid fermentation.

3.6 Effect of Nitrogen source on citric acid production

SSF media of molasses with wheat bran as carrier were supplemented with 1% ammonium sulphate, ammonium nitrate, urea, pepton and yeast extract. The results revealed significant (P≤ 0.05) difference in citric acid production with different nitrogen sources (P=0.003). Yeast extract was found the best nitrogen source which gave maximum yield (17.95 mg/ml) of citric acid. Ammonium sulphate, mmonium nitrate, urea and peptone gave 11.445, 13.840, 13.505 and12.330 mg/ml respectively. Results showed that ammonium sulphate also gave the highest biomass (2.750g) yield followed by ammonium nitrate (2.585g) (Figure 6).

The results are in line with Abou-Zeid and Ashy, who reported that ammonium chloride, ammonium sulphate, peptone and Yeast extract were the most suitable nitrogen addition for production of citric acid by fungus (Abou-Zeid and Ashy, 1984). These results are also in line with Oshoma and Ikenebomeh who found that ammonium sulphate $(NH_4)_2SO_4$ gave the maximum biomass yield of 1.95±0.03 g/l followed by NH_4 NO_3 (1.83±0.04 g/l) (Oshoma and Ikenebomeh, 2005). Ikenebomeh and Chikwendu also reported that $(NH_4)_2$ SO_4 was the best of supplement to improve biomass yield in cassava whey (Ikenebomeh and Chikwendu, 1997). Kareem *et al.* also showed varied effect of type of nitrogen source as ammonium nitrate or phosphate or sulphate (Kareem *et al*., 2010). Kudzai *et al*. (2016) also reported maximum citric

acid production (5.22 g/L) with nitrogen supplement compared to control (4.4 g/L).

3.7 Effect of varying concentrations of best nitrogen source on citric acid production by *A.niger*.

Varying concentrations of yeast extract were added into the flask, experiment was conducted with 1, 2, 3, 4, 5 and 6% of yeast extract. The results showed significant ($P \leq 0.05$) effect of varying concentrations of yeast extract on the citric acid production with different concentrations of yeast extract. It was observed that 1% yeast extract gave the maximum citric acid productivity (mg/ml) and biomass while, higher concentration of yeast extract causes fungal growth inhibition and citric acid production (Figure 7).

These findings were similar to Mirminachi *et al.* who reported limited growth of *A. niger* and increased citric acid production due to limitation or starvation of nitrogen during the fermentation. (Mirminachi *et al*., 2002). Javed *et al.* also reported that concentrations (0.1 to 0.6%) of nitrogen source (ammonium sulphate, peptone and yeast extract) were found to be inhibitory to fungal growth, sugar utilization and citric acid production (Javed *et al*., 2011). Bakhietand Al-Mokhtar also reported that urea as a nitrogen source showed no affect on the citric acid production (Bakhiet and Al-Mokhtar, 2015). The results of present study contrary to Ravindra, who reported that addition of nitrogen supliments improves organism production and hence increase in the biomass cropped (Ravindra, 2001).

3.8 Effect of Surfactants (Tween 80 and SDS)

The results revealed significant ($P \leq 0.05$) difference with respect to the citric acid production with different concentrations of tween 80 ($P = 0.001$) and SDS (P-0.001). It has been observed that Tween 80 and SDS had negligible effect on the citric acid production but addition of these surfactants cause increased production of biomass (Figure 8).

The previous studies revealed that several nonionic surfactants, including Tween 80, Tween 20 and Triton X-100 improve SSF process by microorganisms (*Aspergillus, Trichoderma* or *Nectria*) to improve cellulosic enzyme production such as endoglucanase and exoglucanase and the stimulating effect of surfactants was reported (Goes and Sheppard, 1999). However, in our study, the results showed that tween 80 had no stimulating effect on citric acid production using solid substrate fermentation by *A. niger.*

CONCLUSION

Citric acid can be produced easily by using sugarcane molasses in solid state fermentation with suitable incubation parameters by using microorganism that has the ability to produce citric acid such as *Aspergillus niger*. The result of this study indicates that the use of Sugarcane molasses along with wheat bran to grow fungus for citric acid production represents an operational method of cost reduction and concomitantly producing organic acid of valuable importance.

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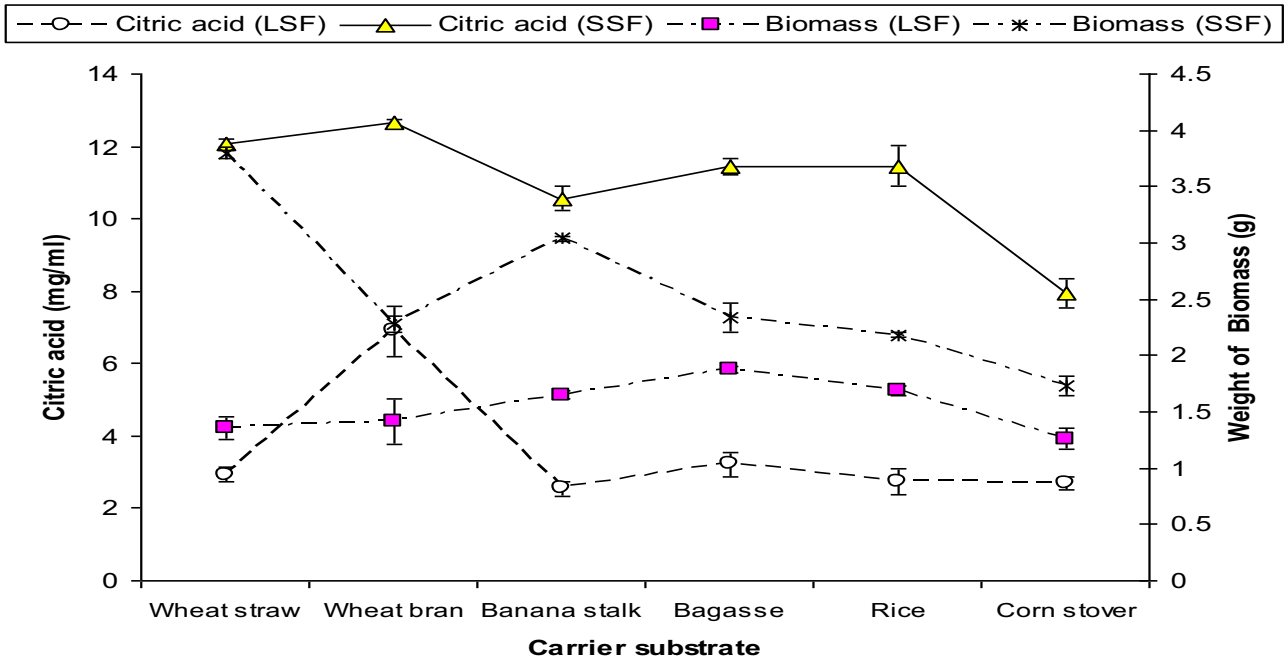
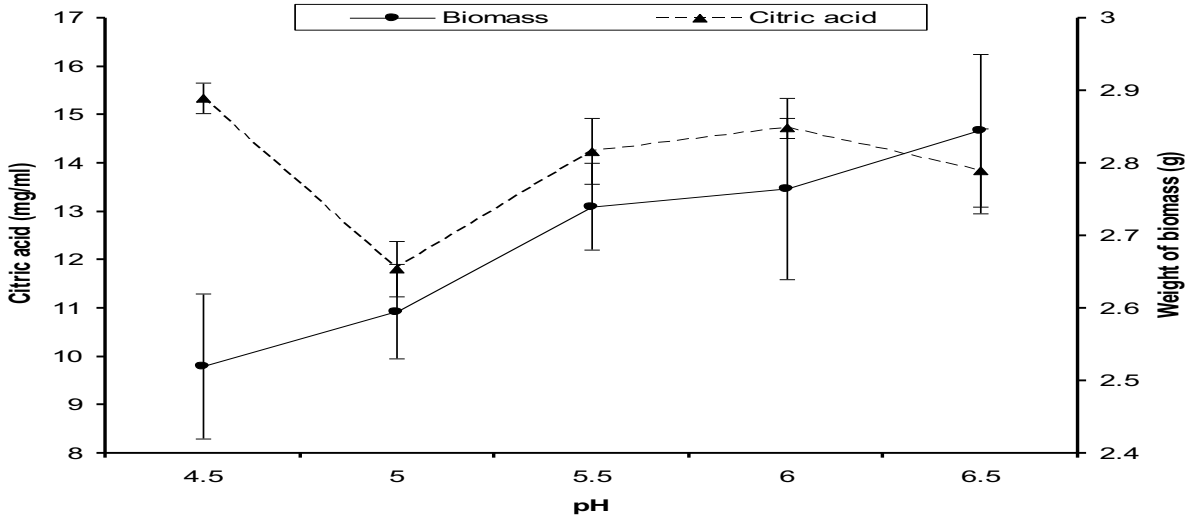


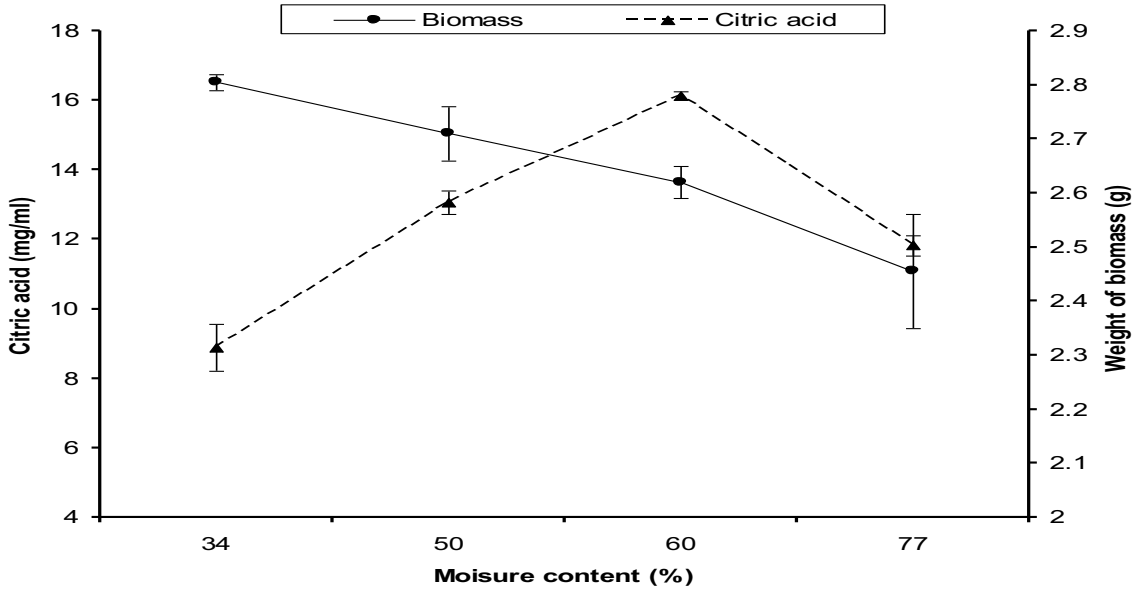
Temperature; 30ºC, pH; 5.5, Moisture level (LSF); 100 ml, Moisture level (SSF); 10 ml
Incubation time; 5 Days

Figure 1: Production of citric acid by *Aspergillus niger* in LSF and SSF of molasses medium using different carrier substrates



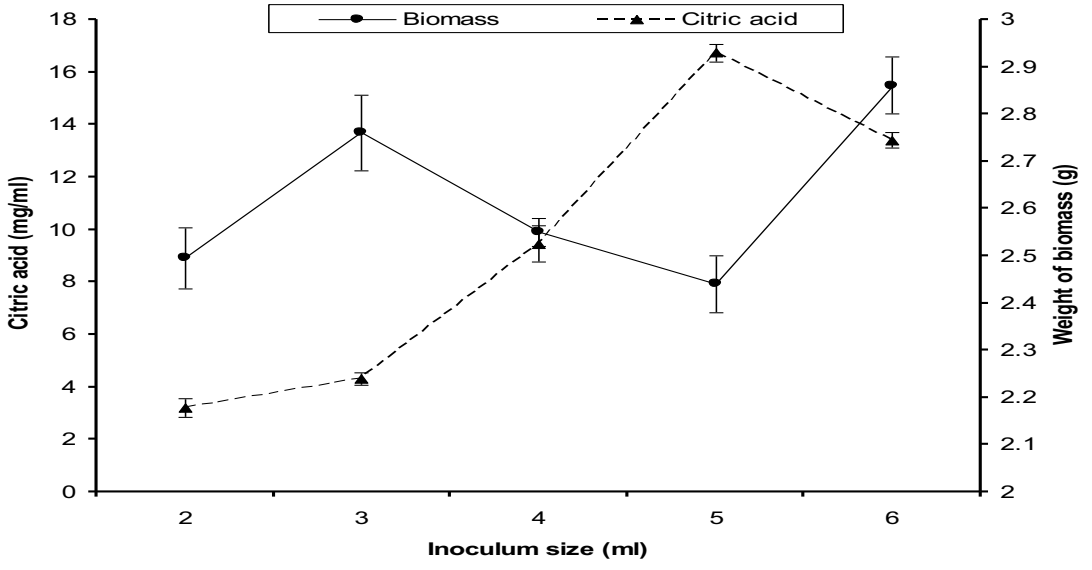
Temperature; 30ºC, Moisture level; 66%, Incubation time; 5 Days

Figure 2: Effect of pH on citric acid and biomass production by *A. niger* in SSF



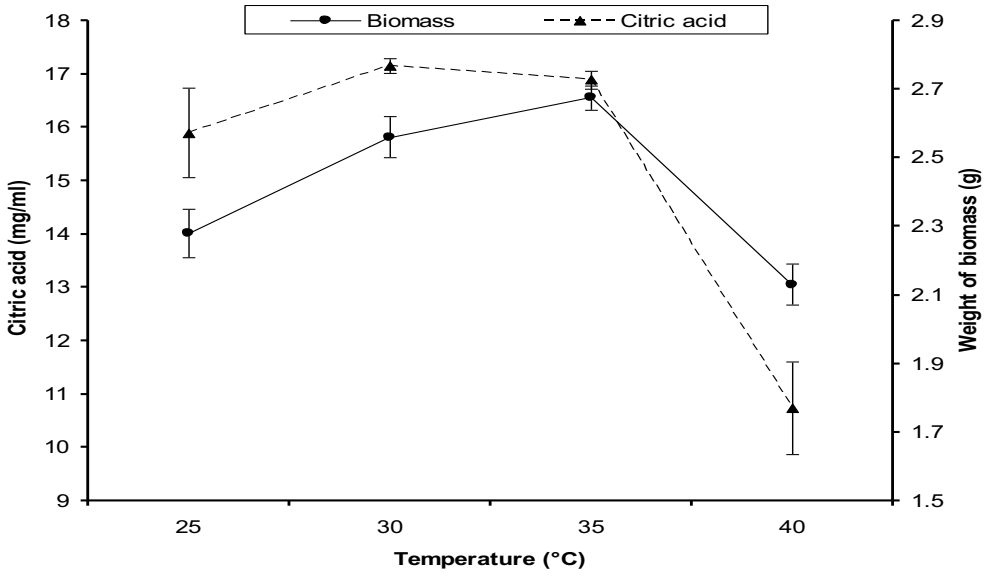
Temperature; 30°C, pH; 4.5, Inoculum level; 5 ml Incubation time; 5 Days

Figure 3: Effect of moisture content on citric acid and biomass Production by *A. niger* in SSF of molasses medium.



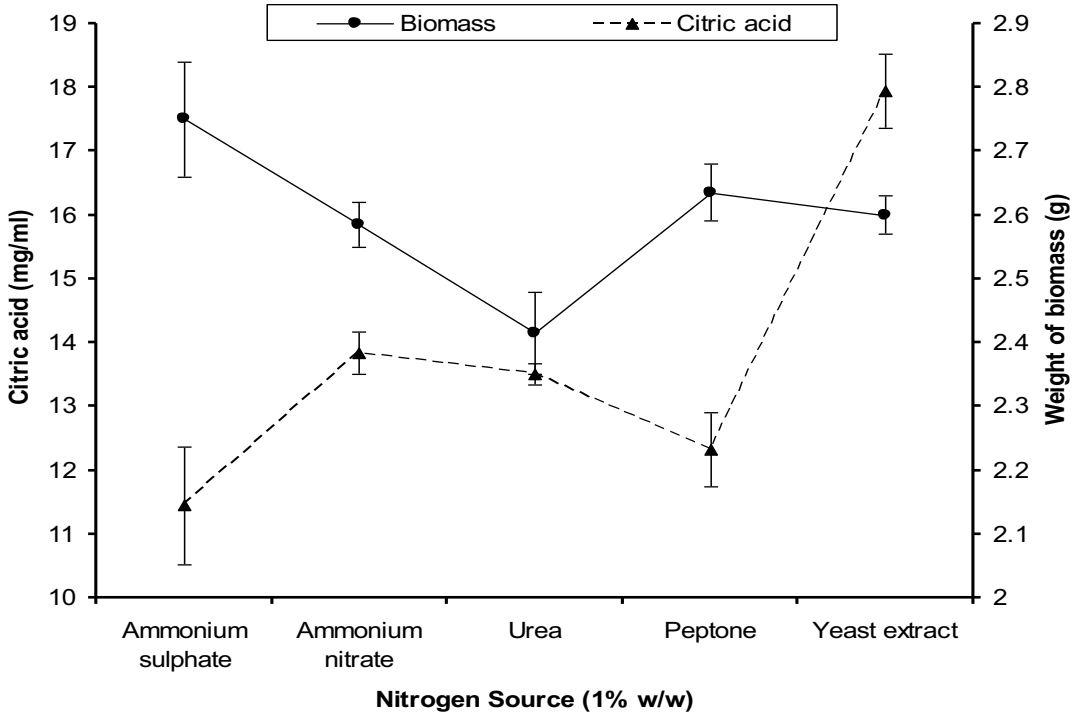
pH.4.5; Moisture level, 8.5 ml; Incubation time, 5 Days

Figure 4: Effect of inoculum level on citric acid and biomass production



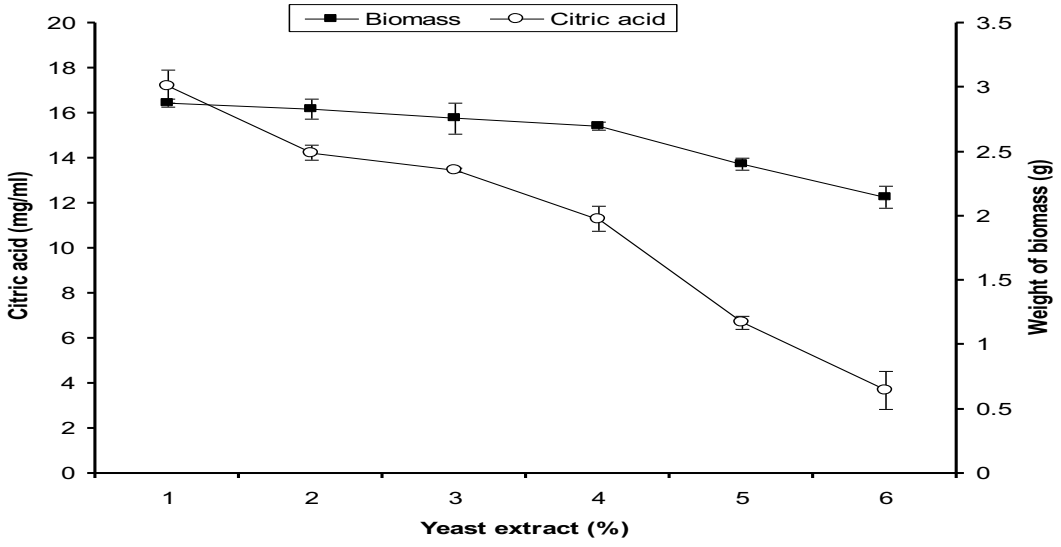
pH.4.5; Moisture level, 8.5 ml; Inoculums level, 5 ml; Incubation time, 5 Days

Figure 5: Effect of temperature on citric acid and biomass production



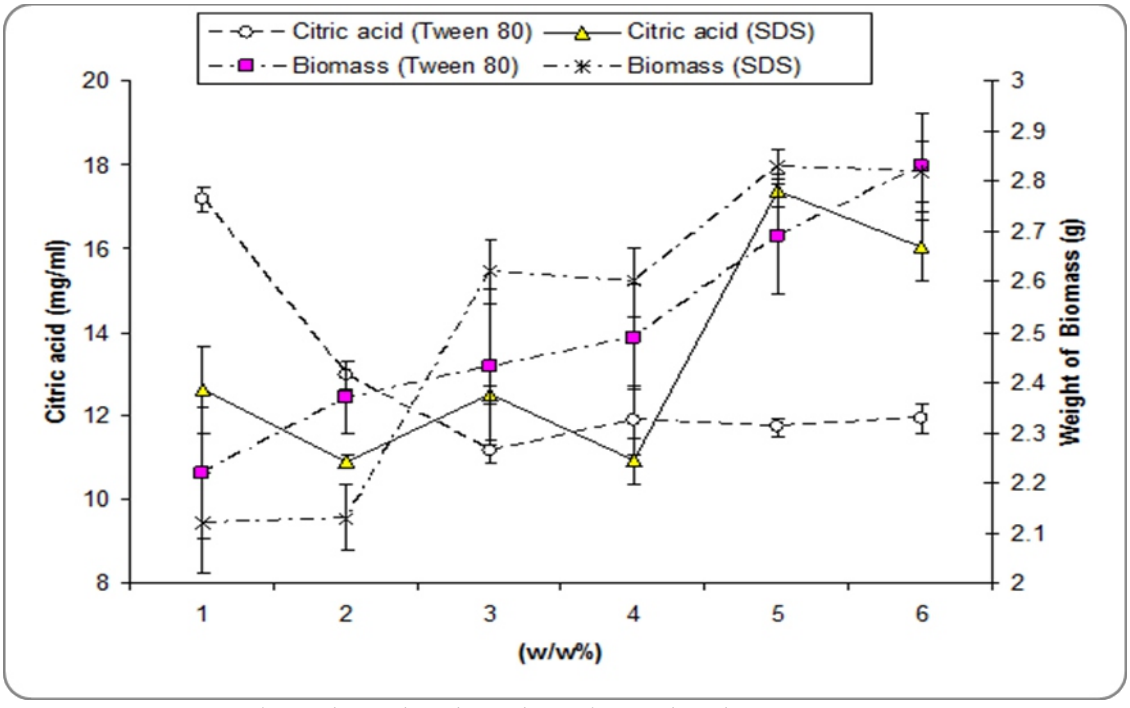
*pH 4.5, inoculums level 5 ml, Moisture level 60%, temp. 30°c, Incubation time; 5 Days

Figure 6: Effect of varying nitrogen supplements on production of citric acid by *A. niger* in SSF under optimum conditions*



pH 4.5, temp. 30°c, inoculums level 5 ml, Moisture level 60%, Incubation time; 5 Days

Figure 7: Effect of varying concentrations of Yeast extract on citric acid and biomass production by *A. niger* in molasses



pH 4.5, temp. 30°c, inoculums level 5 ml, Moisture level 60%, Yeast extract 1%

Incubation time; 5 Days.

Figure 8: Effect of varying concentrations of Tween 80 and SDS on citric acid production by *A.niger* in SSF.