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A comparative study of citric acid production from different agro-industrial wastes by *Aspergillus niger* isolated from mangrove forest soil

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Abstract

Citric acid is an important organic acid having worldwide demand due to its huge application in the food and pharmaceutical industries. To overcome this increasing demand attempt has been made to use cheap agro-industrial waste products as sources of carbohydrate feedstock for citric acid production by fermentation with the fungus *Aspergillus niger*. In the present study, 12 fungal isolates were isolated from soil samples collected from the Bhitarkanika mangrove swamps and named as Bhitarkanika Mangrove Fungi BMF-1 to BMF-12. Out of the 12 fungal isolates, three fungal isolates were identified as *A. niger*. These three fungal isolates were screened for their citric acid production ability and found that the fungal isolate BMF-1 showed the widest yellow zone in Czapek-Dox agar medium, and hence was selected for further citric acid production studies. Fruit peels such as banana peel, rice straw, orange peel and sugarcane bagasses were used for the production of citric acid and compared with the control (sucrose) carbohydrate source of the medium. Maximum citric acid production (0.51%) was obtained with banana peel as a substrate after 6 days of incubation followed by sugarcane bagasses (0.46%), orange peel (0.44%) and rice straw (0.28%) with gradual reduction in pH of the fermentation medium. Maximum reduction in pH (3.3) of the fermentation medium (from initial pH 6.0) was observed after 144 h of incubation with maximum citric acid production by the isolate BMF-1 when banana peel was used as a substrate. To obtain higher citric acid production the fungal growth culture was optimized under different concentrations of banana peel, pH, temperature, nitrogen source, inoculum size and methanol concentration. It has been observed that under optimized condition the production of citric acid was increased from 0.51% to 0.62% which is considered assuitable for citric acid production.

Key words: *Aspergillus niger*, citric acid, fermentation, fungi, mangrove, optimization.
Abbreviations: BMF, Bhitarkanika Mangrove Fungi; PDA, Potato Dextrose Agar.

Introduction

Mangrove forests occurring at the interface between land and sea are a highly productive and biologically diverse ecosystem. This ecosystem is rich in nutrients and harbours diverse groups of microorganisms. (Holguin et al. 2001; Behera et al. 2013). Mangrove vegetation has morphologically and physiologically adapted to harsh conditions such as high salinity, tidal extremes, high wind velocity and anaerobic clayey soils (Thatoi et al. 2013). Mangrove forests are biodiversity "hotspots" for marine fungi (Shearer et al. 2007). Since mangrove vegetation is exposed to various stress conditions, the associated fungi have high stress tolerance capacity (Thatoi et al. 2013). Stress-tolerant fungi inhabiting mangrove habitat are sources for many natural products, which have immense industrial, agricultural and pharmaceutical importance (Thatoi et al. 2013). The microorganisms occur as saprophytes on decomposing organic matter in mangrove ecosystems and play a major role in recycling of various organic waste.

Due to extensive application of citric acid at present time, its demand is very high in the food industry for preserving food, and a demand in the beverage industry, particularly for soft drinks due to its pleasant taste, low toxicity and high solubility in water (Soccol et al. 2006). Various microorganisms have the ability to synthesize citric acid but most of them, however, are not able to produce commercially acceptable yields due to the fact that citric acid is a metabolite of energy metabolism and its accumulation rises in appreciable amounts only under conditions of drastic imbalances (Soccol et al. 2006).

Among all microorganism, the fungus *Aspergillus niger* has remained the organism of choice for commercial citric

acid production due to its ease of handling and high yield of citric acid from a variety of cheap raw material (Soccol et al. 2006). It also produces more citric acid per time unit. Commercial production of citric acid is generally carried out by submerged fermentation of sucrose or molasses, which is costly (Soccol et al. 2006). On the other hand the food and agricultural industry generates a large amount of agro industrial waste during the manufacturing of juice, jellies, jam and pickles, which creates a huge environmental problem regarding recycling to produce valuable commercial products. If these cheap waste materials could be collected and utilized in citric acid production, then the production cost of citric acid and environmental problems could be minimized (Soccol et al. 2003). These agro-industrial residues are very well adapted to fermentation cultures due to their cellulosic and starchy nature, role in solid waste management, the associated biomass energy conservation, production of high value products and little risk of bacterial contamination (Kumar et al. 2002; Kumar, Jain 2008).

The present research work was aimed to investigate the isolation and identification of stress tolerance fungi from stress environment like mangrove and potential application of the isolated fungal strain for citric acid production using different agro-industrial wastes, which could be a good source of raw material to meet the future demand and alternatively could be helpful in reducing environment hazards.

Materials and methods

Isolation and identification of A. niger *from mangrove soil*

Isolation of fungal species was carried out following the standard method of Iqbal et al. (2015). Soil samples were collected from different locations in the Bhitarkanika mangrove swamp. The top layer of soil (about 1 cm) was removed. To isolate fungal colonies, 1 g of air-dried soil sample was diluted up to 10^{-5} in sterilised distilled water and 1 mL suspensions were separately spread plated on petri plates (n = 3) containing Potato Dextrose Agar (PDA) medium (pH 5.6). Streptomycin was added in the molten PDA medium in the Petri dishes and incubated at 30 ± 1 °C for 3 to 5 days. Identification of the *Aspergillus niger* colonies appearing on the agar medium was carried out based on their morphological characteristics and lacto phenol cotton blue staining, following the standard methods of "A Manual of Soil Fungi" (Gilman 1998).

Qualitative screening of citric acid production by A. niger

Qualitative screening of citric acid production by the isolated *A. niger* cultures was carried out following the standard method of Ali (2004). Czapek-Dox agar medium supplemented with bromo cresol purple (10 mL) was poured into individual sterile petri plates in triplicate and allowed to cool at room temperature. Approximately 0.5 mL of the conidial suspension of *A. niger* was transferred to each of the petri plates. The plates were incubated at 30 ± 1 °C for 3 to 5 days. Fungal colonies producing yellow halo zones on the plates were considered as positive for citric acid production (Kareem et al. 2010)

Pre-treatment of substrates

Four different types of agro-industrial waste (banana peels, orange peels, sugarcane bagasses and rice straw) were collected from the local market, washed, air dried and then dried in a hot air oven at 70 °C for about 2 h. Peels were then ground to about 1 to 2 mm size. (Kareem et al. 2010).

Preparation of fermentation media

The composition of original fermentation medium contained (in g L^{-1}): sucrose (140), NH_4NO_3 (2.23), K_2HPO_4 (1.0); $MgSO_4$ (0.23), Cu and Fe compounds (trace amounts), pH 6.0 (Ramesh, Kalaiselvam 2011) After drying at 70 °C to constant weight, the respective types of agro-waste were replaced with the original carbon source (sucrose) of the fermentation medium. The production medium (100 mL) was prepared in 250 mL conical flasks with adjusting the initial pH to 6.0 by 0.1 M NaOH or 1M HCl.

Preparation of spore suspension

The spore suspension was prepared from 7 to10 days old culture of *A. niger* following the standard method of Khattab et al. (2010). Slant cultures of *A. niger* were flooded with 25 mL of sterile saline water mixed with tween 80 (0.1%). Spores were scraped from the surface of the culture slant and further diluted to obtain a desired spore concentration of 2×10^7 spores per mL.

Fermentation

Before inoculation, the fermentation medium was autoclaved at 121 °C for 20 min. After cooling 3% (v/v) methanol was added to the fermentation media to enhance citric acid production (Nadeem et al. 2010). After sterilization, the fermentation medium was inoculated with 0.5 mL of the prepared spore suspension of the selected *Aspergillus* strains and incubated at 30 °C for 7 days by agitating the flasks at 12 h intervals (Dhandayuthapani 2009). All of the runs were performed in triplicate.

Citric acid determination

Every day 10 mL of broth cultures were transfered aseptically from the fermentation medium to a sterile 15 mL falcon tube and final pH of the fermentation medium was recorded daily using a glass electrode pH meter. After pH measurement the broth culture was centrifuged at 5000 rpm for 10 min. The supernatant was removed and citric acid (%) was determined titrimetrically by titration of the filtrate against 0.1 N NaOH using phenolphthalein as indicator (AOAC 1995).

Optimization of citric acid production

Optimization of citric acid production was conducted with respect to different parameters of the culture conditions i.e. temperature, pH, carbon sources, nitrogen sources, inoculums size and methanol concentration and citric acid production was determined titrimetrically following the standard method (AOAC 1995)

Statistical analysis

The results obtained were subjected to statistical analysis as mean and standard deviation (Zar 1984). The mean values and standard deviations were calculated from the data obtained from three different experiments. Statistical difference at $p < 0.05$ was considered to be significant.

Results

Isolation of fungi

A total 12 fungal isolates were isolated and named as Bhitarkanika Mangrove Fungi (BMF) BMF-1 to BMF-12. Of the 12 fungal isolates, three(BMF-1, BMF-3 and BMF-5) showed globose, dark brown conidial heads and spores and were considered as *Aspergillus niger*.

Qualitative screening for citric acid production

All three *Aspergillus* sp. isolated were screened for their citric acid production ability on Czapek-Dox agar medium. After incubation the plates were observed for yellow halo zone production due to citric acid secretion (Fig. 1). Among the three fungal strains, the fungal isolate BMF-1 showed the widest yellow halo zone and has selected for further quantitative estimation studies.

Quantitative estimation of citric acid production

In the present study, different types of agro-industrial waste such as sugarcane bagasses, banana peels, orange peels and rice straw were collected and 70% moisture content was maintained in hot air oven. The dry samples were chopped to a specific size and subjected to submerged fermentation for up to 7 days for comparative study with fresh spore suspension of *A. niger* strain. Maximum citric acid production was observed in the medium supplemented with sucrose without any agro-waste (control). Among the different agro waste types tested, maximum citric acid production (0.51%) was obtained after 6 days of incubation with banana peel as a substrate followed by sugarcane bagasses (0.46%), orange peel (0.44%) and rice straw (0.28%) (Fig. 2). As maximum citric acid production was achieved with banana peel, this substrate hence was selected for further optimization study.

The data obtained from banana peel fermentation were compared with the original composition (sucrose) of the medium. The fermentation was carried out with initial pH of the growth medium 6.0. At every 24 h, the reduction of pH of the medium was also checked and titration of the culture supernatant was carried out with 0.1 N NaOH. Maximum citric acid production was achieved after 6 days of fermentation (Fig. 3A) with maximum reduction of pH of the medium (6.0) in final pH 3.3 (Fig. 3B).

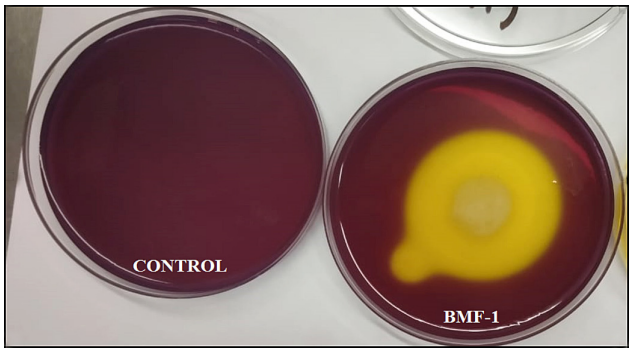

Fig. 1. Qualitative screening indicating production of citric acid by *A. niger.*

Optimization of citric acid production

As the maximum citric acid production by the fungal isolate was observed after 144 h of incubation, the effect of different growth conditions on citric acid production was also observed after 144 h of incubation.

The effect of different concentration of banana peels on citric acid production was investigated and presented in Fig. 4A. In the present study, lower to higher concentration of banana peels was introduced into the production medium to optimise the citric acid production. It has been found that the original quantity (140 g L^{-1}) of the banana peel introduced into the fermentation medium yielded maximum ($p < 0.05$) citric acid production (0.51%) (Fig. 4A). Larger amounts of banana peel affected citric acid production negatively.

A range of initial pH of the medium 2.0 to 7.0 was examined to investigate the effect of pH on citric acid production, keeping the other conditions unchanged. There was maximum ($p < 0.05$) citric acid production observed when the initial pH of the medium was fixed at 6.0 (0.52%;

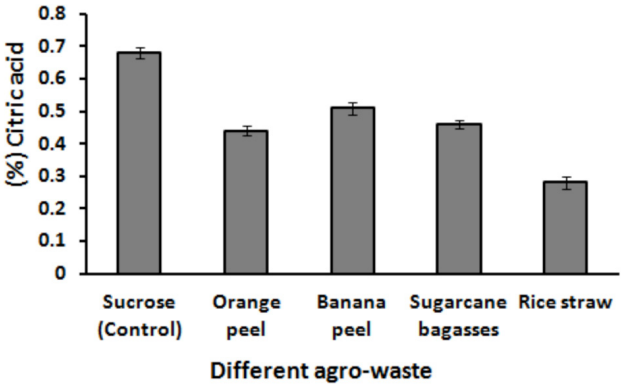

Fig. 2. Quantitative estimation of citric acid production by *A. niger* with different types of agro-industrial waste.

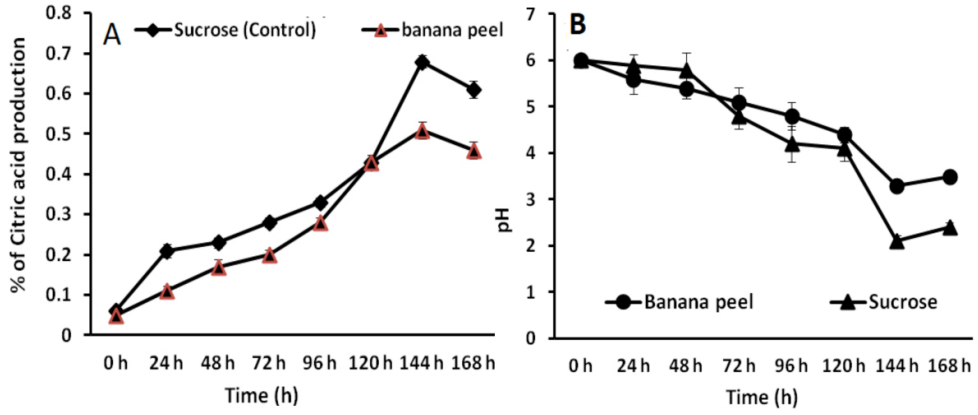

Fig. 3. Citric acid production at different time period (A) and reduction in pH of the fermentation medium at different time period (B).

Fig.4B). Further increase or decrease of initial pH of the fermentation medium from pH 6.0 was associated with a decrease in citric acid yield.

The fungal isolate produced maximum citric acid (0.53%) at 30 °C (at 6 days of incubation, pH 6.0). A significant decline in citric acid production was observed at higher temperature (Fig. 4C). All experiments were run parallel in triplicates and values differed significantly at $p < 0.05$.

Different nitrogen sources were supplied under the above optimized conditions in the broth medium to increase citric acid production. The fungal isolate BMF-1 showed maximum citric acid production (0.53%), when NH_4NO_3 was supplemented into the medium (Fig. 4D).

In the present study maximum citric acid production (0.56 %) was achieved with 4% (v/v) methanol concentration in the fermentation medium (Fig. 4E). An increase or decrease in methanol concentration caused a decrease in citric acid production activity.

The influence of various concentrations of *A. niger* spores on citric acid fermentation was quantified. With the previously optimized growth conditions, the effects of various concentrations of fungal spore suspension or inoculum size in a range of 125 to 1000 µL (v/v) were tested for optimum citric acid production. The maximum amount of citric acid production (0.62%) was observed when 750 µL of inoculum was supplemented (Fig. 4F) in 100 mL of the production medium. A larger or smaller inoculum size reduced citric acid production.

Discussion

In the present study *A. niger* isolated and identified from mangrove soil was evaluated for citric acid production.

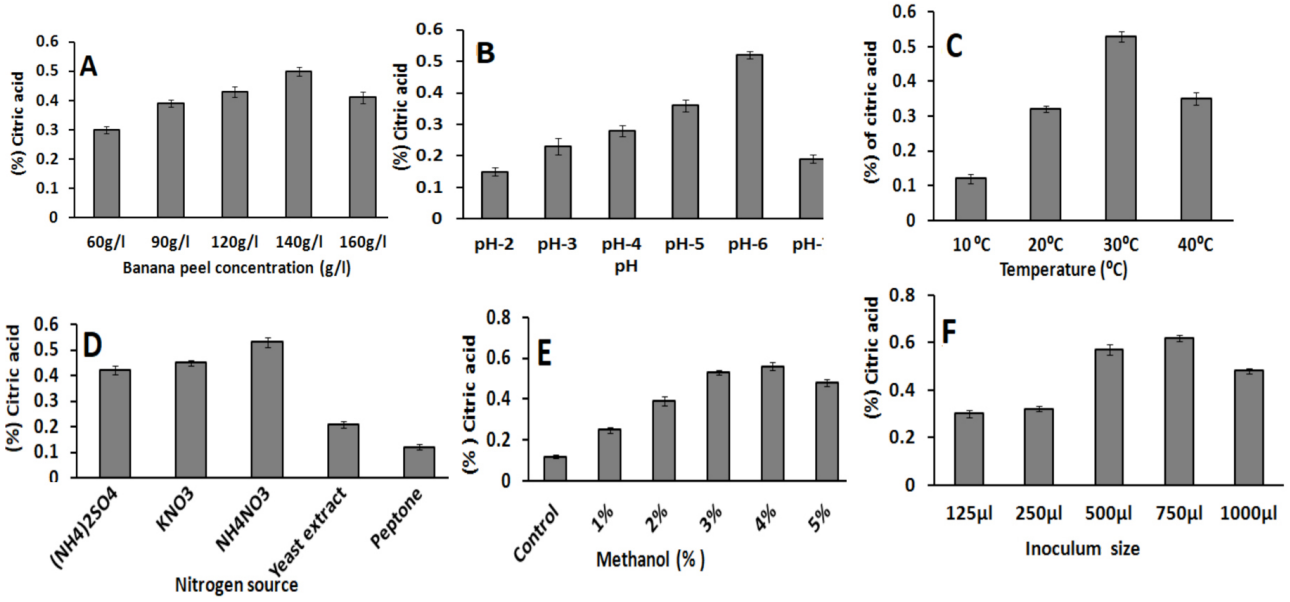

Fig. 4. Optimization of citric acid production with different concentration of banana peel (A), various initial pH of the fermentation medium (B), temperature (C), nitrogen source (D), methanol concentration (E) and inoculum size (F).

Isolation of *A. niger* species from soil samples of the Bhitarkanika mangrove swamp, Odisha has been reported earlier (Mishra et al. 2010; Behera et al. 2012)

Three *Aspergillus* sp. isolated were screened for their citric acid production ability on Czapek-Dox agar medium. Although the actual mechanism of the screening procedure is not understood completely, it is known that the structure of bromo cresol purple, which is an indicator dye, changes with pH. When *A. niger* produces citric acid in the medium it diffuses through the agar medium and hence reacts with the dye and changes the purple colour of the dye to yellow (Ali et al. 2002).

Quantitative estimation showed that maximum citric acid production (0.51%) was obtained after 6 days of incubation with banana peel as a substrate followed by sugarcane bagasses (0.46%), orange peel (0.44) and rice straw (0.28%). These levels are higher than these observed by Pathania et al. (2018) and Bhattacherjee and Baruah (2015), who observed maximum citric acid production of 0.23 and 0.33% respectively by *A. niger*. In contrast, the present finding is lower than that reported by Iralapati and Kummari (2014): 1.15% for banana peel and 0.51% for orange peel as substrate by *A. niger*. The present findings are in agreement with Laboni et al. (2010), who reported that the citric acid production increased with the increase of the fermentation period but decreases after longer period. This decrease in citric acid production may be due to the age of fungi, decreased available nitrogen in the fermentation medium, inhibitory effect of high concentration of citric acid, depletion of sugar contents and decay in the enzyme system responsible for citric acid biosynthesis. The maximum citric acid production in the present investigation was observed after 6 days of incubation which supports the observation of Ali et al. (2002) and Wieczorek and Brauer (1998), who also reported maximum citric acid production after 6 days of fermentation period.

During fermentation there was a gradual reduction in pH, which was noticed in all the experiments and related to the production of citric acid. These findings are in confirmation with Thangavelu and Kalaiselyam (2011) who observed reduction in pH of the production medium from pH 6.5 to 1.5 during fermentation. As maximum citric acid production was achieved with banana peel as a substrate after 6 days of incubation it was selected for further optimization study using different experimental conditions such as different pH, temperature, carbon source, nitrogen source, methanol concentration and inoculum size.

Citric acid accumulation is strongly influenced by the type and concentration of concentration of the carbon sources (Soccol et al. 2003). High concentrations of the carbon leads to suppression of α-ketoglutarate dehydrogenase and results in high citric acid production. In the present study, the effect of increasing substrate (banana peel) concentration on citric acid production was estimated by changing the substrate concentration keeping other factors unchanged. The maximum amount of citric acid (0.51%) obtained in the study was at 140 g L^{-1}. When the substrate concentration was higher than 140 g L^{-1}, citric acid production decreased. The decrease in citric acid production at concentration higher than 140 g L^{-1} soluble substrate may be due to polyalcohol formation (Nwoba et al. 2012). This concentration is higher than that reported by Bhattacherjee and Baruah (2015) who observed 0.38 g per 100 mL of citric acid at optimum carbon concentration but lower than the observation of Shankar and Shivkumar (2016). A higher initial sugar concentration resulting in increase in citric acid yield and productivity was also reported by Khosravi and Zoghi (2008).

The initial pH of the medium is an important factor for citric acid fermentation. Maximum citric acid production was observed at initial pH 6.0. There was decrease in citric acid production when the initial pH of the medium was either increased or decreased from 6.0. This would seem to suggest that the initial pH did not directly influence the citric acid production mechanism, but rather affected the enzymes which were active in degrading substrate or the permeability of the cell membrane to the substrate (Nwoba et al. 2012). The present work is in agreement with earlier reports (Bhattacharjee, Baruah 2015; Shankar, Sivakumar 2016; Khattab et al. 2017) who reported maximum citric acid production when initial pH of the growth medium was fixed at 6.0. Maximum citric acid production at initial pH 5.5 (Al-Shehri and Mostafa 2006; Nwoba et al. 2012), at initial pH 5.0 (Khattab et al. 2017), and initial pH 3.5 (Pandy et al. 2013) with respect to different substrates have been reported. Fermentation starts from inoculation of 5 to10 days spore inoculums and the germination of these spores requires an initial pH of medium above 5 (Max et al. 2010). Gradual lowering the pH of the fermentation medium during fermentation improves citric acid production, which might be due to consumption of ammonia by germinating spores and subsequent release of protons (Max et al. 2010). During the fermentation, the final low pH of the medium minimizes the risk of contamination of other microorganisms, thus inhibiting oxalic acid biosynthesis, which makes recovery of citric acid difficult (Max et al. 2010). Hence initial pH before fermentation and final pH during the fermentation are important factors affecting the citric acid production.

Temperature is an important factor for microbial growth and organic acid production. Temperature was found to influence extracellular secretion, possibly by changing the physical properties of the cell membrane (Jansova et al. 1993). In the present study, a significant decline in citric acid production was observed above the optimum temperature (30 °C; Fig. 4C). When the temperature of the medium is above 30 °C the biosynthesis of citric acid is lower due to the accumulation of the by-products such as oxalic acid (Kubicek and Rohr 1986). Citric acid production at 30 °C was also reported earlier (Ali et al. 2002; Kim et al. 2002;

Kareem et al. 2010). Similarly, Roukas (1999) reported that citric acid concentration increased significantly with an increase in the fermentation temperature from 25 to 30 °C and decreased at higher temperature. In contrast, Szewczyk and Myszka (1994) found that temperature did not have a strong effect on the growth rate of *A. niger* within a range of 28 to 34 °C in solid- state fermentation. Similarly the present result is in agreement with that obtained by Auta et al. (2014) who recorded an optimum temperature of 30 °C for citric acid production by *A. niger* using *Parkia biglobosa* fruit pulp as substrate.

Source of nitrogen is also one of the important factors that affects the metabolism of microorganisms and variation in the nitrogen source can affect metabolic processes of cells significantly (Kulkarni et al. 1999). Nitrogen sources are also necessary to maintain the pH of the fermentation medium. High nitrogen concentrations increase fungal growth and sugar concentration, but decrease citric acid production (Vanderberghe et al. 1999). In comparison to peptone, yeast extract, $(NH_4)_2SO$, and KNO_3, the nitrogen source NH_4NO_3 showed significantly higher citric acid production. The present study is in agreement with others (Chirova et al. 2016; Shankar, Shivakumar 2016) who reported maximum citric acid production with NH_4NO_3 as a nitrogen source. However, peptone (Pathania et al. 2018), $(NH_4)_2SO_4$ (Bhattacharjee, Baruah 2015) and $NaNO_3$ (Khattab et al. 2017) were also reported as the best nitrogen sources for citric acid production. Jennison et al. (1955) found that intrinsic differences in the molecular structure may be involved in the differences in utilization of these nitrogen compounds

Use of low molecular weight alcohols i.e. methanol and isopropanol as adjuncts to the culture medium can greatly increase citric acid production (Moyer 1953). Low methanol concentrations are usually necessary to eliminate the adverse effect of trace metals, whereas high concentrations are used for highly contaminated materials (Rivas et al. 2008). However, the addition of excess alcohol was shown to be inhibitory when added to synthetic broths (Prescott and Dunn 1959). *A. niger* does not assimilate methanol and although its exact role in the stimulation of citric acid production is not yet known, as it is believed that methanol increases the permeability of the microorganism cell membrane, thereby making the excretion of citric acid easier (Kapoor et al. 1982). In the present study maximum citric acid production was achieved with 4% (v/v) methanol concentration in the fermentation medium (Fig. 4E). An increase or decrease in methanol concentration from this level caused decrease in citric acid production activity. Our finding supports the study of Kumar et al. (2002) who observed maximum citric acid production at 4% (v/v) methanol concentration in the fermentation medium. An increase in citric acid production with application of methanol was in agreement with Hossain et al. (1984) who stated that the presence of methanol in fermentation media may increase citric acid production by *A. niger*. The effect of methanol on citric acid production may be due to reduction of the inhibitory effects of metal ions (Kiel et al. 1981).

The influence of various concentrations of *A. niger* spores on citric acid fermentation was quantified. The maximum amount of citric acid production was observed when 750 µL of the inoculum was added into 100 mL of the production medium. This is a lower concentration than that reported by Khattab et al. (2017), who observed maximum citric acid production at 1% inoculum in suspension. In addition maximum citric acid production at much higher (5%) inoculum concentration was also reported by Maharani et al. (2014). Increase or decrease in inoculum concentration reduced citric acid production. The initial increase in the number of inoculated spores gradually increases the level of acid production. However, a dense population consumes the limited nutrients of the fermentation medium, thus resulting in reduction in citric acid production (Dhillon et al. 2013). A much low level of inoculation also causes prolongation of the adaptation phase, and therefore decrease the final yield (Alam et al. 2011).

Increasing demand for citric acid in various applications has led to exploration of means for its efficient production. In view of the negative impact of agro-industrial waste disposal on the environment and on the other hand the escalating cost of citric acid production, it is necessary to explore alternative organic substrates for cost-effective production. The present study showed great potential for comparative study of different available agro-industrial waste as a substrate for the production of citric acid by *A. niger,* which can certainly aid in the reduction of production costs associated with costly synthetic substrates. Use of these agro wastes for the production of citric acid may have the combined benefit of utilising a low-grade waste while producing a commercially valuable product. Based on the work done so far, it can be concluded that among the different agro industrial waste used in the study, banana peel is an abundant agricultural waste material that may be used as a cheap alternative medium for the effective production of citric acid, which can alternatively reduce the cost of citric acid production.

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