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EFFECT OF GROUNDNUT CAKE AND SOYA BEANS ON ENHANCED CITRIC ACID PRODUCTION FROM PAWPAW AND ORANGE PEEL BY MUTANTS OF *ASPERGILLUS NIGER*

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ABSTRACT

This present study was concerned with the biosynthesis of citric acid (CA) with mutant strain of *Aspergillus niger* using pawpaw and orange peel as substrates by solid state fermentation process. The *A. niger* strain isolated from spoilt orange was identified, screened for CA production on Czapek-Dox Agar and subjected to mutation by ethidium bromide. The effect of carbon sources, nitrogen sources and substrates were also determined. Among the mutant strains, *A. niger PJ-02* A_{120} was found to be the best mutant that produced citric acid (65.00 ± 0.58^{f}) after 48 hours in Vogel's medium. The effects of carbon sources (sucrose and glucose) on CA production from each substrate (orange and pawpaw peel) using mutant *A. niger PJ-02* was determined and sucrose, the best carbon source was combined with two the nitrogen sources (groundnut cake and soyabeans) to determine the most suitable supplement for CA production. Groundnut cake enhances the production of citric acid while soyabeans was inhibitory. Citric acid was further produced in pawpaw peel and orange peel medium containing sucrose (5 %) groundnut cake (2 %), methanol (1.5 %) and the mutant strain. The orange peel substrates yielded 112.07g/kg of CA while 107.17g/kg was recorded for pawpaw peel when fermented for 5 days at 30°C. The Production of citric acid with mutant *Aspergillus niger* proved better with orange peel than pawpaw peel when optimized with alcohol.

Key words: *A. niger PJ-02,* ethydium bromide, orange peel (OP), mutant, methanol, pawpaw peel.

INTRODUCTION

Citric acid fermentation as an industrial fermentation process requires biotechnological advancement in production for global sustainability using improved strains of fermentable microorganisms as starting materials.

These improvements can be carried out by mutagenesis and selection of parental strains using mutagens. *A. niger* is the most commonly used fungus for the production of citric acid due to the high yield and relatively high tolerance to acid accumulation (Dhillon *et al*., 2010). Consequently, the production of citric acid depends on the use of an appropriate strain, along with adequate aeration, carbon source, nitrogen, phosphate, appropriate pH and trace elements. Mutation is a process by which the characteristics of a strain are improved physiologically and morphologically. Chemically induced mutagene-

sis has been shown to alter the cell wall composition in filamentous fungi which may significantly affect citric acid production (Vasanthabharathi *et al.*, 2013).

Orange peels and pawpaw peels are rich in cellulosic materials. They also constitute low sugary substrates and the organism producing citric acid utilizes these cellulose and sugars efficiently to produce citric acid. Hence, this manuscript reports the enhanced citric acid production by mutant strains of *A. niger*, using selected carbon and nitrogen sources on pawpaw and orange fruit peels.

MATERIALS AND METHODS

Sample collection

Pour-plate method was adopted to obtain pure cultures of fungal isolates from decayed fruit sample. Pure cultures were obtained by subculturing and maintained on agar Potato Dextrose Agar (OXOID) (Nwoba *et al*., 2012).

Screening and selection of strain

Czapeck dox agar with bromocresol green (0.14 gm/l) as the indicator was inoculated with the fungal isolates and incubated at ±28°C for 72 hours for detection of yellow zone around the mycelia of the isolates (Ali, 2004; Kareem *et al*., 2010; Amal, 2018).

Molecular characterization of the highest citric acid producing isolate

The fungal isolates were identified by DNA extraction, gel electrophoresis, polymerase chain reaction (PCR) and sequencing of the extracted DNA.

DNA extraction

DNA extraction was done with ZR fungal/bacterial DNA extraction kit. The mold isolates were subcultured on Potato dextrose broth and incubated at 30 °C for 72 hrs. The aliquot of each test mold organism was dispensed into Microcentrifuge tube (Eppendorf tube) and centrifuged at 13.3 revolutions per minute (rpm) for 10minutes, and repeated until enough pellet was obtained. The pellets were then washed by introducing sterile distilled water. The extracted DNA was stored at -20°C for PCR.

Polymerase chain reaction (PCR)

PCR amplification of ITS with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') was conducted on a Thermo Cycler. 5µM of each primer, 2µl genomic DNA and nuclease free water was adjusted to a final volume of 50µl. Initial denaturation at 94 °C/5mins, 30 cycles of 72° C/45secs, 56 °C/30secs and final extension/elongation at 72 °C/5mins. The isolate was purified with absolute ethanol and 70% ethanol respectively. Finally, 2.5ul of the purified amplicon was used for sequencing. (White *et al.*, 1990).

Sequencing of the 18s rDNA genes of the fungal isolate

The purified DNA was sequenced by Bioscience Centre, International Institute of Tropical Agriculture, Ibadan, Nigeria, using the big dye 3.1 kit and ABI 3500XL genetic analyser. DNA sequences were imported from Bioedit to NCBI database. A genetic similarity search was performed by aligning the sequence products into BLAST (Basic Local Alignment Search Tool).

Mutagenesis assay/screening and selection of mutants

Vogel's medium was prepared and added into test tubes containing *A. niger* spores. One ml of ethidium bromide was added into those test tubes for incubation of the spores.Each derivative from exposure to

mutagen for thirty, sixty, ninety, one hundred and twenty, one hundred and eighty minutes respectively were designated as A_{30}, A_{60}, A_{90}, A_{120}, A_{150} and A_{180} and screened in a bromocresol-Czapek dox agar medium at 30 °C for 12 hrs, 24hrs and 48 hrs respectively, using a modified method of Tehreema *et al*., (2010). The mutants were selected based on the changes observed in the morphology of the colonies or sporulation on PDA, the treated strains appredrd whitish and wooly compared to the control (without exposure to ethidium bromide) and the citric acid production. The purified mutants were further screened for the production of citric acid.

Solid state fermentation process

The solid state fermentation media were prepared by adding 5g each of the substrate into two different 250 ml conical flasks in triplicates, moistened to 70% and sterilized at121^{o} C for 15 mins and then inoculated with 1 ml spores suspension of the mutant strain to serve as the control of experiment. The effects of glucose and sucrose as carbon sources at 1g, 3g and 5g on the substrates were subsequently studied. Groundnut cake and soyabeans were added at 1g, 1.5g, and 2g respectively as nitrogen sources into the substrates with the selected carbon source moistened, sterilized and incubated at 28^{o}C for 5 days. It was optimized with different concentrations of methanol and ethanol from 0.1%-5% w/v. Each medium was inoculated with 1ml of mutant *Aspergillus niger* suspension and incubated in a rotary shaking incubator for 5 days, then analyzed for citric acid production. 2x10^{7} spores/ml of the inoculum was used.

Citric acid recovery and determination

The crude citric acid was recovered from each of the mouldy substrates using the modified method of Pratik*et al*., (2013). The slurry was filtered through Whatman filter paper no.1 to separate the biomass from the fermentation broth. The filtered extracts were used as the crude citric acid source. Citric acid (CA) was determined titrimetrically, using the formula below:

$$\%CA = \frac{\text{Normality} \times \text{volume of NaOH} \times \text{Equiv. wt. of CA} \times \text{dilution factor}}{\text{Weight of sample} \times 10}$$

Kareem *et al*., (2010)

RESULTS

The fungal isolate from rotten fruit identified as *A.niger PJ-02* and earlier screened for high citric acid production was exposed to Ethidium bromide at different time interval and re-screened with Czapek dox agar with bromocresol green for the rate of citric acid production as seen in Table 1

Table 1: *A. niger PJ-02* mutants zones of colouration (mm) in relation to time (hrs) for citric acid screening

Mutants	12 hrs	24hrs	48 hrs
A_{30}	31.00±0.58[a]	35.00±0.58[a]	41.00±0.58[a]
A_{60}	35.00±0.58[b]	40.00±0.58[c]	46.00±0.58[c]
A_{90}	36.00±0.58[b]	48.00±0.58[e]	56.00±0.58[e]
A_{120}	38.00±0.58[c]	52.00±0.58[f]	65.00±0.58[f]
A_{150}	38.00±0.58[c]	44.00±0.58[d]	50.00±0.58[d]
A_{180}	32.00±0.69[a]	39.00±0.58[b]	43.00±0.58[b]

*Mean of triplicate determinations

Fig 1 presents the citric acid produced by mutant *A.nigerPJ-02*on orange peel (OP) medium supplemented with glucose and sucrose respectively as carbon supplements.

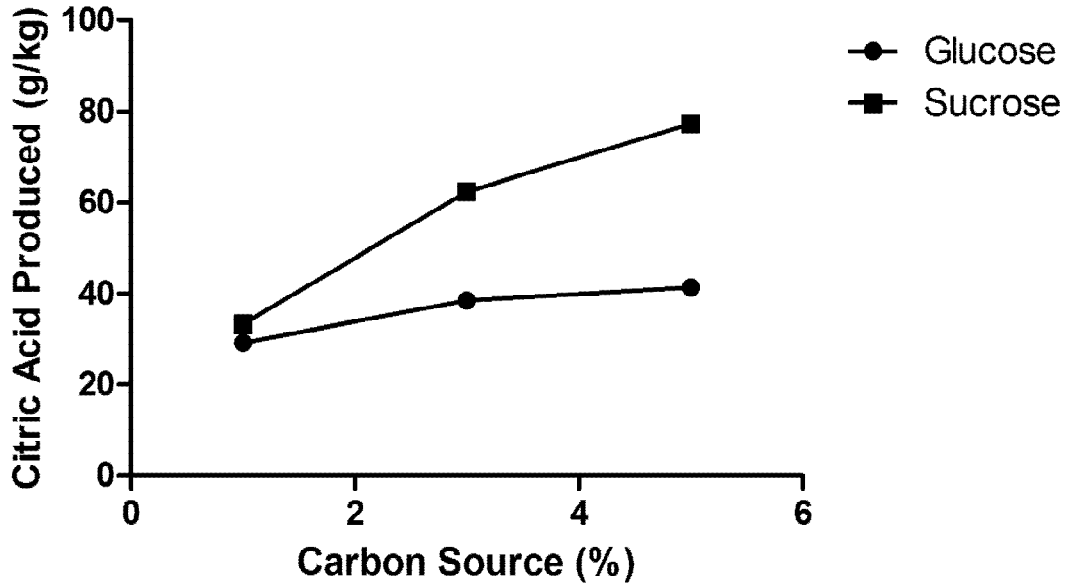

Fig 1: Effect of carbon sources on orange peel (OP) medium

Citric acid production by mutant *A. nigerPJ-02*from pawpaw peels as a fermentation medium with the different concentrations of sucrose and glucose is shown in Fig 2.

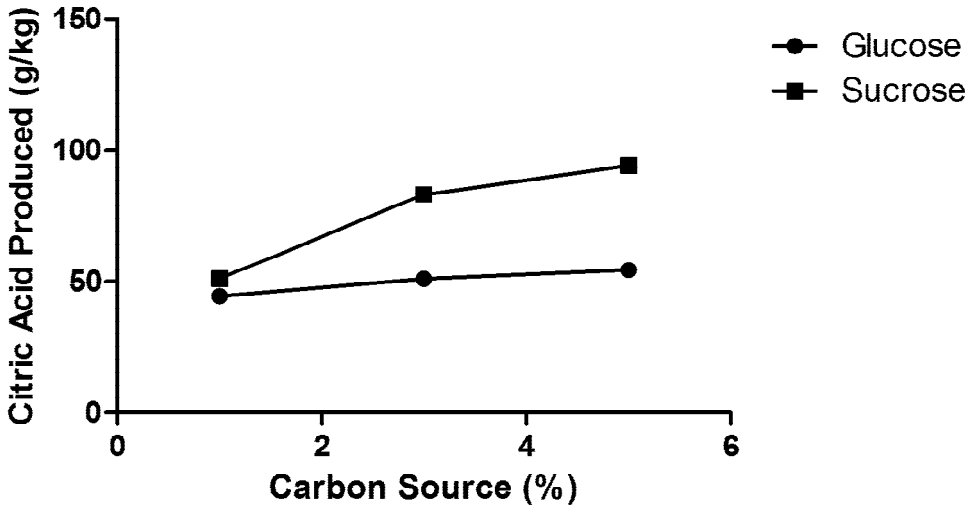

Fig 2: Effect of carbon sources on pawpaw peel (PP) medium

The effect of nitrogen sources on orange peel medium supplemented with sucrose (OS) for citric acid production by mutant *A.niger* is shown in Fig 3.

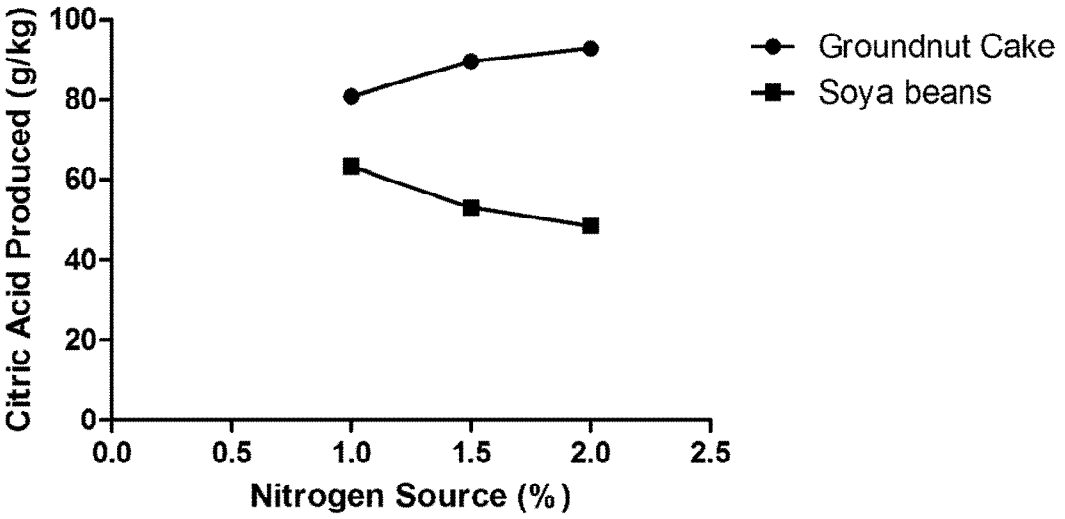

Fig 3: Effect of nitrogen sources on orange peel with sucrose (OS) medium

The effect of nitrogen sources on pawpaw peel medium with sucrose for citric acid production by mutant *A.nigerPJ-02* is presented in Fig4.

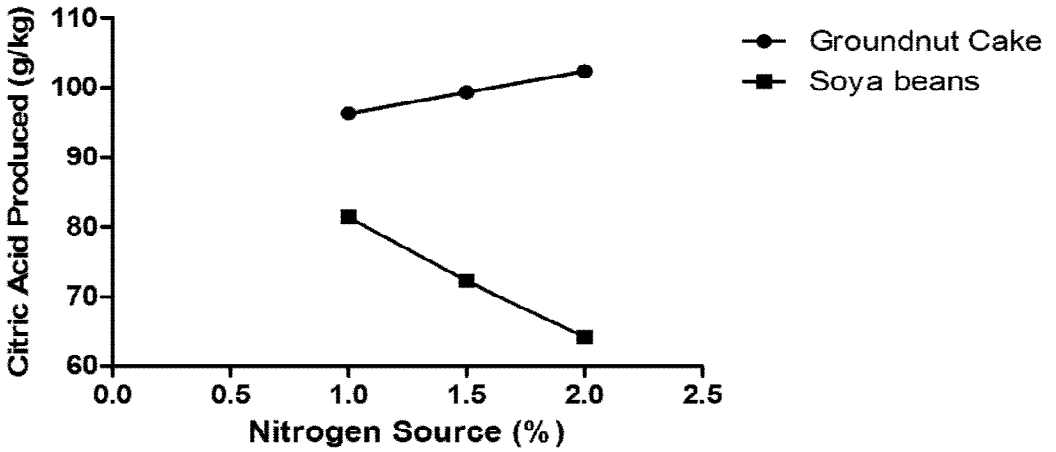

Fig 4: Effect of nitrogen sources on pawpaw peel with sucrose (PS) medium

The effect of methanol on citric acid produced from OSG and PSG is presented in Fig 5

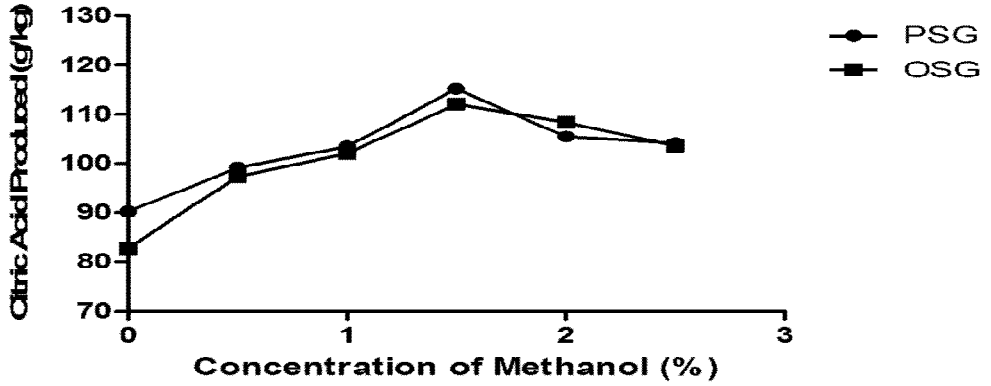

Fig 5: Effect of methanol on orange peel with sucrose and groundnut cake (OSG) and pawpaw peel with sucrose and groundnut cake (PSG) media

The effect of ethanol on citric acid produced from OSG and PSG is presented in Figure 6

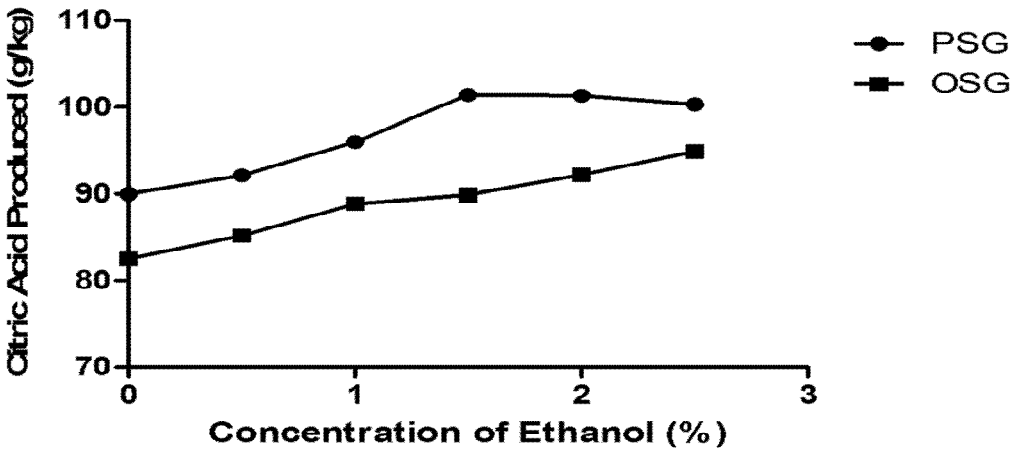

Fig 6: Effect of ethanol on orange peel with sucrose and groundnut cake (OSG) and pawpaw peel with sucrose and groundnut cake (PSG) media

DISCUSSION

A successful production of citric acid depends on the strain of microorganism, nitrogen source, carbon source and the substrate. This study shows the effect of ethidium bromide on *A. niger PJ-02* for optimum citric acid production by solid state fermentation.There was a varying yellow zone of colouration around the mycelia of each mutant derivative and the morphology of the colonies or sporulation on PDA. The highest citric acid producing mutant observed after 48 hrs of incubation on Czapek-dox agar is in accordance with the result of Javed *et al*., (2011) whose ethidium bromide treated mutants exposed for 12 producers.

Carbon sources play a significant role in the production of citric acid. The complexity of the carbohydrates has been reported to be crucial to the biosynthesis of citric acid (Soccol et al., 2006). Generally, sugars that allows for easy uptake by the mould gives more citric acid yield. Of the two carbon sources supplemented with the substrates sucrose gave a higher citric acid value over glucose with mutant strain at the same concentration after 5 days of fermentation. This agrees with the study of Amenaghawon *et al.,* (2013), who reported sucrose to be a better carbon source the production of citric acid production. Higher citric acid yield was also reported by Amal *et al*. (2018) with sucrose as a carbon source than glucose. The maximum production of citric acid observed from pawpaw peel is expected to be as a result of the enhanced carbohydrate content in the pawpaw peel substrate due to the pulp content in the peel.

Addition of groundnut cake to both substrates increased citric acid production by mutant *A. nigerPJ-02* while addition of soyabeans gave a reduced value. The addition of soyabeans to both substrates alongside sucrose in this study was inhibitory to citric acid production. Rao and Reddy, (2013) reported a fall in citric acid production as the concentration of ammonium nitrate was gradually increased. Javed *et al.* (2011) on the

contrary, showed enhanced citric acid production by A. *niger* EB-3 in the presence ammonium nitrate along and sugar.

The reduction in citric acid production from both orange peel with sucrose and groundnut cake (OSG) medium and pawpaw peel with sucrose and groundnut cake (PSG) medium at 2% methanol concentration from the two media is in agreement with the report of Belen *et al.,* (2010) which found out that methanol can be detrimental to citric acid production if the microorganism can no longer absorb it, thus causing a decline. The increase in ethanol concentration on PSG with the mutant strain became insignificant at 2%. This is similar to the observation by Ashraf *et al.,* (2004) whose addition of ethanol after accumulation of citric acid to 40.65g/kg became insignificant thus giving no increase in citric acid production. In contrast, production of citric acid increased on OSG as the concentration of alcohol increased to 2.5%.

CONCLUSION

The study established pawpaw peel and orange peel as a good agricultural waste for solid state fermentation media in citric acid production using mutant *A.niger PJ-02*. Orange peel with sucrose and groundnut cake as carbon and nitrogen sources of choice proved a better substrate for citric acid production, when optimized with 1.5%w/v of methanol. This study equally indicated that the citric acid yield from orange peels and pawpaw peels are efficient substrate supplement for citric acid production.

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