Int.J.Curr.Microbiol.App.Sci (2016) *5*(5): 483-492

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 5 (2016) pp. 483-492**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.505.050

Citric Acid Production from Different Sources under Submerged Conditions using *Aspergillus niger*

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Keywords
Citric acid, *Aspergillusniger*, Submerged fermentation, Czapek dox, Cane molasses, Orange peel, titration, Optical density, pH and temperature.

Article Info

Accepted:
18 April 2016
Available Online:
10 May 2016

A B S T R A C T

Extensive developments in the area of industrial biotechnology over past few years have signified the importance of citric acid as one of the most known organic acid, applied mainly as flavoring, emulsifying, cleaning, and chelating agent. It also plays a major role in pharmaceutical and cosmetic industries. Citric acid is produced by various microorganisms such as *Aspergillus niger*, *Penicillium restrictum*, *Trichoderma viride*, *Mucor piriformis*, *A. awamori*. *A. niger* is one of the most commonly used source for citric acid production under submerged fermentation conditions. *A.niger* was isolated from rotten onion, coconut, and lemon and was grown in supplemented Potato Dextrose Broth (PDB) at 30°C for 7 days. Best source for isolation of *A.niger* amongst these sources was analyzed by observation of optical density. Subsequently, production of citric acid using A.Niger on variable sugar sources was carried under Nitrogen and phosphorus limitation. Three different production media for citric acid production were used,Cane Molasses, Czapek Dox and Orange peel media. Effect of variation of pH in the range of 1-5 and temperature in the range of 200 to 500C over production of citric acid in cane molasses, Czapek Dox and Orange peel was estimated. Quantitative estimation of citric acid produced under varying environmental parameters in submerged culture condition was done using titration method and it was observed the best production media for citric acid production using A.nigerwas Cane molasses media at the pH 1 and temperature 20°C.

Introduction

Citric acid i.e. 3-carboxy-3-hydroxy pentane-1-5-dioic acid (C6H8O7) is a weak organic acid found naturally in citrus fruits such as limes, oranges, berries, tangerines, and grape fruits. It is an intermediate compound formed in Kreb'scycle (TCA Cycle) and is a common metabolite in all aerobic organisms. Industrially, citric acid is produced by submerged fermentation. The first industrial production of citric acid began in 1890 by Italian Citrus Fruit industry, calcium citrate was precipitated by treating the juice with calcium hydroxide (hydrated lime), then this calcium citrate

was converted to citric acid using sulfuric acid. Two forms of citric acid have been observed, one is anhydrous form (water-free form) and other is monohydrate form. The later can be converted to anhydrous form at 78°C.Millions of tons of citric acid is produced per year. Dominant use of citric acid is mainly in food (70%), in pharmaceuticals (12%), others (18%), (Tran *et al.*, 1998; Wang 1998; Ates *et al.*, 2002). According to recent studies and developments citric acid based polymers have been created with enhanced mechanical properties used in the development of biodegradable elastomers which has been increasingly important for tissue engineering, for culturing variety of cells, in many biomedical applications and in drug delivery (Richard T. Tran, Yi Zhang, DipendraGyawali and Jian Yiang, 2009). Common applications of citric acid is as flavor enhancers, preservatives, as chelating and emulsifying agent. It is also a common constituent in kitchen cleaning solutions. In military, citric acid is used for efficient removal of post soldering flux residues (Robin *et al.*, 1995; Ashkan *et al.*, 2010; Guillermo *et al.*, 2010).

Citric acid is produced by various microorganisms such as *Aspergillus niger*, *Penicillium restrictum*, *Trichoderma viride*, *Mucor piriformis*, *A. awamori*, but production of citric acid by *A. niger*is one of the most commonly used organism using submerged fermentation. *A.niger* is generally considered as a asexual organism but, sexually reproducing spores also have been found hence it is classified in class Deuteromycetes, it belongs to the phylum Ascomycota, class Eurtiomycetes, order of Eurotiales, family of Trichocomacae and genus Aspergillus. Morphologically, it appears black long conidiophores with globose at the tip. Being ubiquitous in nature it can grow on diverse habitats such as rotten vegetables and fruits, soil and plant debris and can be identified by producing colonies with carbon black conidia. The advantage of *A. niger* over other citric acid producing microorganisms is that it can grow on nutrient depleted media, it is thermo-tolerant to low temperatures and gives high yield. Recent research concluded that by increasing the spore count from 104 to 109 spores per ml as inoculum results in the bigger *A.niger* cells that can be used for enzymatic production of chitosan (Javedchehri, 2013).

Countries like Brazil, Bangladesh import citric acid from other countries and due to its extensive use in day to day life its demand is increasing. Citric acid can be produced chemically but production by microbial fermentation has proven to be a better option (Mattey, 1992). Studies reveal mutant strains of *A. niger* (GCBT7) shows hyper-producibility of citric acid without supplements (Sikander Ali, Ikram-ul-haq, Qadeer, Javed Iqbal, 2002). Keeping in view, the advantage of biological fermentation over chemical synthesis we had choosen three media Czapek dox, orange peel, and cane molasses media. More than one substrate used for fermentative media had been proven to be best for citric acid production (Laboni Majumder *et al.*, 2010).

The objective of this study was to identify the best source for isolating the *Aspergillus niger*, Assess the impact of different environmental conditions i.e. temperature and pH on citric acid production and, estimate the best production media for citric acid production using *A. niger*.

Materials and Methods

Isolation of *Aspergillus niger*: Potato Dextrose Broth(PDB) was prepared by dissolving 7.2 g of PDB in 300 ml of distilled water and was autoclaved at 121°C

for 20 minutes. Further PDB media was divided equally into 3 flasks. *A. niger* was isolated from three different sources, rotten coconut, onion, and lemonon PDB media by inoculating 1 gm of each source. Inoculated samples were kept at 30°C for 7 days at 80 rpm in orbital shaker for incubation. Growth of *A. niger* was analyzed by measuring Optical density at 580nm in spectrophotometer.

Plating was done to obtain colonies of *A. niger* using Czapek Dox agar. Six plates were prepared, two for each sample. Streaking was done and plates were maintained at 30°C for 5 days in B.O.D. incubator. Maximum growth was observed in plates streaked with onion sample, hence concluded onion is the best source for *A. niger* isolation. Seed culture was prepared by taking the colonies from plate streaked with onion sample in 200 ml of distilled water with 4.8g of PDB and was kept in orbital shaker at 30°C for 2 days for acclimatization.

Substrate used: (a) Czapek dox broth (b) Cane molasses (c) Orange peels

Pre-treatment of substrate and preparation of media:

Orange Peel Media

Orange peels were washed with tap water, cut in small pieces and dried in oven at 60°C for overnight. The substrate was powdered by using a mortar pestle and then sieved. Dried powder of orange peel (150g) was mixed with 150g glucose, 150g sucrose, 2.5g NH4NO3, 5g (NH4)3PO4, 0.25g/l MgSO4.7H2O in 1000ml of distilled water. This 1 liter solution was equally divided into 5 flasks each containing 200ml of solution and pH was varied from 1 to 5. All the flasks were then autoclaved.

Cane Molasses Media

Cane molasses media was prepared by boiling 5-6 glasses of sugarcane juice for few hours and was concentrated to 100ml solution. 35ml of 1N H2SO4 and 900 ml of distilled water was added to 100 ml of the solution to make the solution volume upto 1000ml.This solution was again boiled for half an hour. After cooling, solution was neutralized with calcium oxide and kept overnight. Clear supernatant liquid(800ml) was pipette out and addition of distilled water(200ml) was done to make the volume upto 1000ml .Further addition of NH4NO3 2.5g, (NH4)3PO4 5g, MgSO4.7H2O 0.25 g/l to solution.. This 1litre solution was equally divided into 5 flasks each containing 200ml of solution with variation in pH ranging from 1 to 5 and then all flasks were autoclaved.

Czapek Dox Media

Czapek dox broth was prepared by addition of NaNO3 3g, K2HPO4 1g, MgSO4.7H2O 0.5g, KCl 0.5g, FeSO4 0.01g, Sucrose 30g in 1000ml of distilled water and pH variation (1 to 5) was done by dividing 1 liter solution equally into 5 flasks containing 200 ml each and then autoclaved.

Inoculation and Incubation

Cane molasses media with pH=1 (200ml) was further subdivided into 4 flasks each containing 50ml media were inoculated with seed culture (2ml) each and all 4 flasks were kept at different temperatures i.e. 20°C, 30°C, 40°C, and 50° C in different incubators for 12 days. Same was done for pH 2, pH 3, pH 4 and pH 5. Similar procedure was applied to other two, Czapek dox and Orange peel media.

Estimation method

Mycelial wet weight was determined using filtration and centrifugation method and mycelial mat was kept in oven for overnight at 70°C to determine the dry weight also.

Citric acid Estimation

Estimation of citric acid was done by titration method by using 0.1N NaOH for cane molasses and czapek dox and 1N NaOH for Orange peel media with phenolphthalein as indicator.

% citric acid was calculated by using following formula:

% citric acid = N×V1×EqWt / V2 × 10

where;
N = Normality of NaOH solution
V1 = Volume of 0.1N NaOH for cane molasses and czapek dox / Volume of 1N NaOH for orange peel media.
EqWt. = Equivalent weight of citric acid.
V2 = Volume of sample (ml)

Results and Discussion

After isolation of *A.niger* on PDB media, maximum optical density was observed on Onion i.e. 1.6, however, it was close to growth on Lemon , optical density being 1.5. Minimum optical density was observed on Coconut sample. It indicates that Onion and Lemon are the best sources to extract and isolate *A.niger* under temperature conditions of 30ºC. However, V. Maharani (et al 2014) have reported spoiled coconut is prominent source for *A.niger* at pH 3.5 and temperature 30º C.

As per the estimation of wet weight of *A.niger* on cane molasses media, Best growth is observed at pH 2, temperature 30ºC. The growth was observed around 5.04g, and minimum was observed at pH 3 and temperature 50ºC. While, the estimation of wet weight of *A.niger* on orange peel media revealed, best growth at pH 1 and temperature 40ºC, where the growth was around 5.46g, and minimum growth was observed at pH 5, temperature 50ºC. Calculation of wet weight of *A.niger* on Czapek dox media indicated the best growth at pH 5 and temperature 20ºC, being 8.127g, whereas the minimum growth was observed at pH 5 and temperature 50ºC.

As per the observation related to the % production of citric acid on Cane Molasses media, the highest amount was detected at pH 1, Temperature 20ºC, being 51.6%, whereas the lowest being estimated at pH 1 and temperature 40ºC. Calculations show the maximum production of citric acid on Orange peel media was observed at pH 1 and temperature 30ºC being 51%, and the lowest was recorded at pH 4, temperature 30ºC. While, Czapek dox media indicated the highest citric acid production to be at pH 1 and temperature 50ºC, being 19.5%, whereas the lowest being estimated at pH 1 temperature 30ºC.

Table.1 Optical Density of *A.niger* on Different Sources:

Sample	O.D. (580nm)
Coconut	1.17
Lemon	1.5
Onion	1.6

Table.2 Biomass of *A.niger* on Cane Molasses Media at varying pH and Temperature

pH	Temp (°C)	Wet wt. (g)	Dry wt.(g)
1	20	3.706	0.223
	30	3.390	0.297
	40	0.101	0.393
	50	0.716	0.300
2	20	1.024	0.352
	30	5.041	0.413
	40	4.382	0.470
	50	0.985	0.327
3	20	1.897	0.367
	30	2.430	0.390
	40	2.536	0.477
	50	0.060	0.023
4	20	2.839	0.543
	30	3.930	0.252
	40	1.900	0.047
	50	0.607	0.334
5	20	2.930	0.018
	30	2.112	0.660
	40	2.437	0.563
	50	0.502	0.379

Table.3 Biomass of *A.niger* on Czapek Dox Media at varying pH and Temperature

pH	Temp (°C)	Wet wt. (g)	Dry wt.(g)
1	20	0.049	0.009
	30	5.620	0.489
	40	0.061	0.013
	50	0.023	0.000
2	20	3.657	0.451
	30	4.480	0.344
	40	3.334	0.316
	50	0.025	0.000
3	20	4.643	0.442
	30	6.360	0.244
	40	5.704	0.282
	50	0.310	0.000
4	20	3.952	0.461
	30	5.421	0.285
	40	4.801	0.340
	50	0.021	0.000
5	20	8.127	0.684
	30	5.850	0.309
	40	5.090	0.271
	50	0.018	0.000

Table.4 Biomass of *A.niger* on Orange Peel Media at varying pH and Temperature

pH	Temp (°C)	Wet wt. (g)	Dry wt.(g)
1	20	3.340	0.546
	30	3.482	0.849
	40	5.460	0.933
	50	3.005	0.907
2	20	2.035	0.387
	30	3.573	0.384
	40	1.167	0.304
	50	3.300	0.674
3	20	2.547	0.300
	30	2.130	0.420
	40	3.458	0.759
	50	0.923	0.377
4	20	2.677	0.520
	30	2.219	0.672
	40	2.207	0.720
	50	1.868	0.906
5	20	3.822	0.559
	30	3.204	0.515
	40	2.259	0.358
	50	0.891	0.176

Fig.1 % Citric Acid production on Cane molasses Media

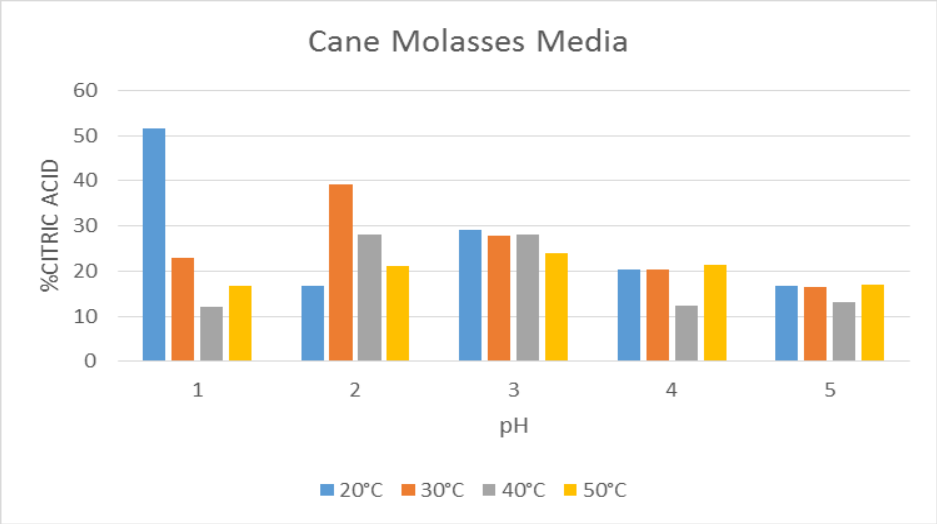

Table.5 % Citric Acid produced on Cane Molasses Media at Variable pH and Temperature.

Media	pH	Temp(°C)	% acid(w/v)
Cane Molasses Media	1	20	51.6
		30	22.8
		40	12.0
		50	16.8
	2	20	16.8
		30	39.2
		40	28.2
		50	21.0
	3	20	29.1
		30	27.9
		40	28.2
		50	24.0
	4	20	20.4
		30	20.4
		40	12.3
		50	21.3
	5	20	16.8
		30	16.4
		40	13.2
		50	17.1

Fig.2 % Citric Acid production on Czapek dox Media

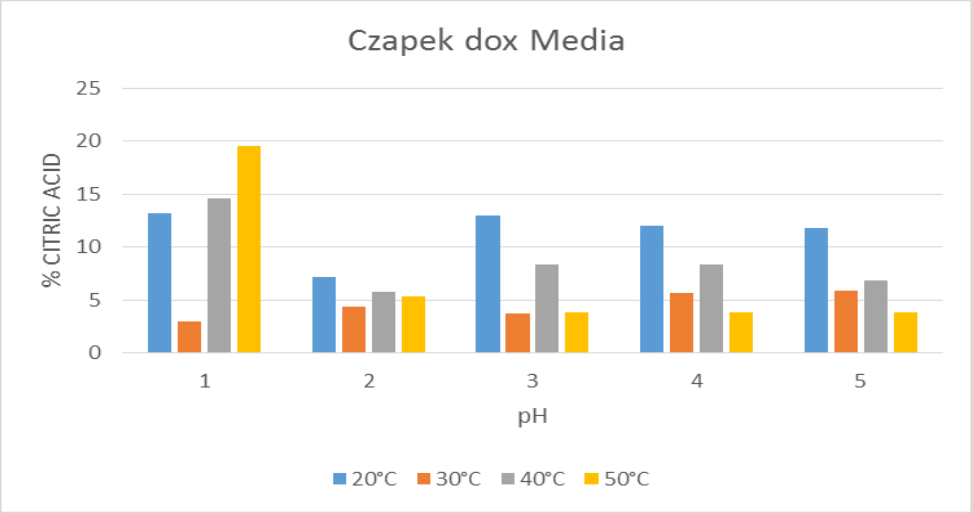

Table.6 % Citric Acid produced on Orange Peel Media at variable pH and Temperature.

Media	pH	Temp(°C)	%acid(w/v)
Orange Peel Media	1	20	24.0
		30	51.0
		40	27.0
		50	30.0
	2	20	18.0
		30	18.0
		40	24.0
		50	30.0
	3	20	3.0
		30	24.0
		40	27.0
		50	30.0
	4	20	21.0
		30	15.0
		40	17.0
		50	24.0
	5	20	21.6
		30	24.0
		40	22.8
		50	34.2

Fig.3 % Citric Acid production on Orange Peel Media

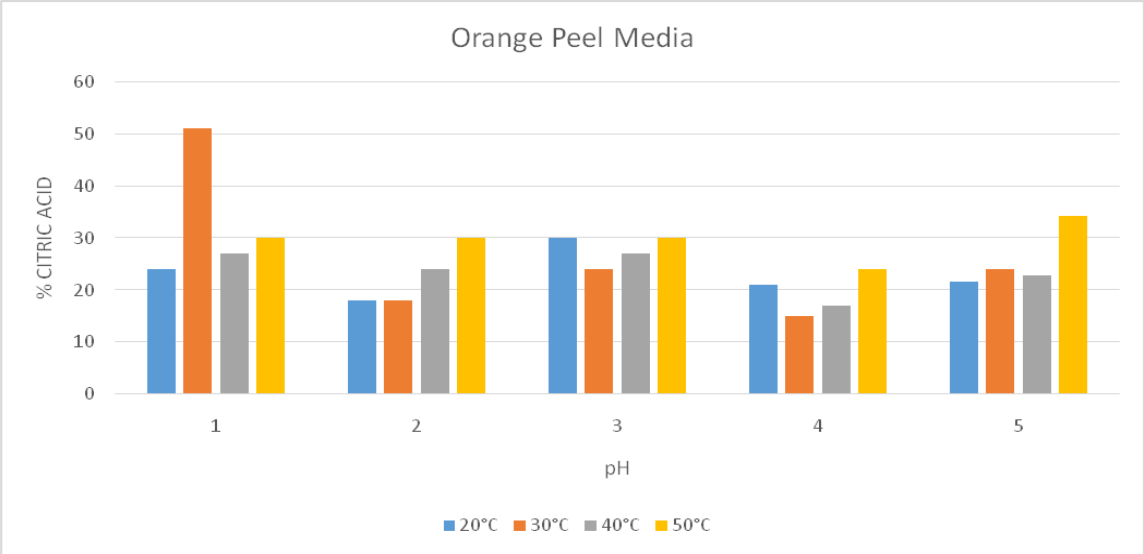

Table.7 % Citric Acid produced on Czapek Dox Media at variable pH and Temperature.

Media	pH	Temp(°C)	%acid (w/v)
Czapek Dox Media	1	20	13.2
		30	3.00
		40	14.6
		50	19.5
	2	20	7.20
		30	4.40
		40	5.76
		50	5.40
	3	20	13.0
		30	3.70
		40	8.40
		50	3.84
	4	20	12.0
		30	5.70
		40	8.40
		50	3.90
	5	20	11.8
		30	5.88
		40	6.90
		50	3.90

Current findings of carried research work suggest cane molasses and orange peel media to be best sources for extraction of citric acid at pH 1 and temperature 25°C to 30°C temperature. The conclusion of our work is coherent with the findings of other research groups which have suggested that lower the pH of media, better is the production percentage of citric acid on industrial scale.

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How to cite this article:

Pallavi Singh, Sadiya Draboo, Aishwarya Singh, Swapnil Chaturvedi, Swati Sharma and Pooja Verma. 2016. Citric Acid Production from Different Sources under Submerged Conditions using *Aspergillus niger*.*Int.J.Curr.Microbiol.App.Sci.* 5(5): 483-492.
doi: http://dx.doi.org/10.20546/ijcmas.2016.505.050