ORIGINAL ARTICLE

Comparison of citric acid production from *Aspergillus niger* in solid and suspension state fermentation

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Abstract

Aim: Due to huge demand of citric acid, there is a need to find alternatives for its efficient production either using low-cost substrates or by improving the potency of the fermentation microorganisms. **Materials and Methods:** In this study, microbial production of citric acid was performed by *Aspergillus niger*. Solid and suspension state fermentation was carried out using biodiesel waste, i.e. the oilseed cake of *Pongamia pinnata* (honge) as a substrate for the comparative study of citric acid production using *A. niger*. After the estimation, the concentration of citric acid was found to be more in suspension state fermentation than in solid state. Hence, the further analysis was carried out using suspension culture. In the first stage, weight was varied, and the optimum weight was found out to be 12.5 g. Then keeping this optimum weight as constant, the inoculum size was varied, also keeping the optimum pH 6 and temperature 32°C constant. The rpm of suspension state was kept 100. Through this, optimum inoculum size was obtained. **Results and Discussion:** The yield of citric acid in solid state fermentation (SSF) was found to be 4.74 mg in 12.5 g of substrate and suspension state was 9.6 mg/ml. **Conclusion:** Suspension state fermentation method gives a higher yield of citric acid using oil seed cake as the substrate from *A. niger* than SSF method. The highest yield of 10.43 mg/ml of citric acid was obtained by taking substrate weight 12.5 g and inoculum size $275*10^4$ spores/ml by suspension state fermentation method.

Key words: *Aspergillus niger*, citric acid, estimation and analysis, oilseed cake substrate, solid and suspension sate fermentation

INTRODUCTION

Organic acids have a long history of being utilized as food additives and preservatives for preventing food deterioration and extending the shelf life of perishable food ingredient. The main organic acids in industrial use are citric, acetic, tartaric, malic, lactic, and gluconic acid. The most utilized organic acid is citric acid. The food industry is the largest consumer of citric acid, using almost 70% of the total production followed by about 12% for the pharmaceutical industry and 18% for other applications.[10]

Submerged Fermentation (SmF)

The SmF process is the commonly employed technique for citric acid production. It is estimated that about 80% of world production is obtained by SmF. Several advantages such as higher yields and productivity and lower labor costs are the main reasons for this. Two types of fermenters, conventional stirred fermenters and tower fermenters are employed, although the latter is preferred due to the advantages, it offers on price, size, and operation. Preferentially, fermenters are made of high-grade steel and require the provision of aeration system, which can maintain a high dissolved oxygen level. Fermenters for citric acid production do not have to be built as pressure vessels since sterilization is performed by simply steaming without applying pressure. Cooling can be done by an external water film over

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the entire outside wall of the fermenter. In SmF, different kinds of media are employed such as sugar and starch based media.[2]

Solid State Fermentation (SSF)

SSF has been termed as an alternative method to produce citric acid from agro-industrial residues. Citric acid production by SSF (the Koji process) was first developed in Japan and is as the simplest method for its production. SSF can be carried out using several raw materials. In general, the substrate is moistened to about 70% moisture depending on the substrate absorption capacity. The initial pH is normally adjusted to 4.5–6.0 and the temperature of incubation can vary from 28 to 30°C. The most commonly organism is *Aspergillus niger*. However, there also have been reports with yeasts. One of the important advantages of the SSF process is that the presence of trace elements may not affect citric acid production as it does in SmF. Consequently, substrate pre-treatment is not required.[2]

MATERIALS AND METHODS

Substrate Used

The substrate we used is *Pongamia pinnata* oilseed cake. Also known as honge, these seeds are used in making biodiesel. After the extraction of oil, the cake what is left out is powdered and used as the substrate for the growth of *A. niger*. The cake is rich in nitrogen, phosphorous, potassium, and carbon which act as the nutrient source for the growth of the *A. niger*.

Culture Preparation

Pure culture of *A. niger*was subcultured using solid media and liquid media.

Inoculum Preparation

Sterile discs were smeared on the culture of *A. niger* to get an equal amount of spores on each side.

Spore Count

One disc was smeared with *A. niger* and dipped in distilled water and mixed properly. 1 ml of this sample was taken, and the spore count is done using hemocytometer.

Spores/ml = A+B+C+D* dilution factor

General Procedure

About 250 ml of potato dextrose broth (PDB) is proposed and sterilized. Keep the flask with PDB in the laminar air flow. Sporulating culture of *A. niger*is inoculated into a potato broth aseptically. The flask is then incubated at room temperature for 6–7 days. The pH of the media is then checked for citric acid production.

Substrate Collection and Preparation

Honge oil seed cake used as the substrate was collected from biodiesel center, Nitte. The cake was grounded using a mixer, and the powder was stored in an airtight container. The ground waste will be taken in 250 ml conical flask and moistened with distilled water to desired moisture level. The flasks containing medium will be sterilized at 121°C for 1 h to provide proper cooking to the substrate and to increase its amenability for the microbe. After sterilization, flasks containing medium will be allowed to cool to room temperature.[1,5]

SSF

5 g, 7.5 g, 10 g, 12.5 g, and 15 g of the substrate were varied and added to 5 conical flasks, respectively. Distilled water was added to maintain the moisture content. The flasks containing the substrate were sterilized at 121°C for an hour. Then, it was allowed to cool at room temperature. 4 discs smeared with a pure culture of *A. niger* was dropped into the flasks in the laminar air flow. Then, it was incubated at 32°C for 6 days.

Suspension State Fermentation

5 g, 7.5 g, 10 g, 12.5 g, and 15 g of the substrate were varied and added to the conical flasks, respectively. The suspension was prepared by adding 100 ml of distilled water. The pH of the substrate was checked and maintained to pH 6 by addition of 0.1 M sodium bicarbonate. The flasks were sterilized at 121°C for an hour. Then, it was allowed to cool at room temperature. 4 discs smeared with a pure culture of *A. niger* were dropped into the flasks in the laminar air flow. Then, the flasks were kept in the rotatory shaker at 100 rpm and incubated at 32°C for 6 days.[7]

Varying the Inoculum Size Carried by Suspension State Fermentation

Optimum weight of the substrate was taken in all the flasks. The suspension was prepared by adding 100 ml of distilled water and sterilized. The pH 6 was maintained by addition of 0.1 M sodium bicarbonate. 2 discs, 3 discs, 4 discs, 5 discs, and 6 discs smeared with *A. niger* were dropped into the 5 flasks, respectively. It was kept in the rotatory shaker at 100 rpm and incubated at 32°C for 6 days.

Extraction and Analytical Methods

Fermented material of the flask will be dried in an oven at 50°C and extracted by the addition of 100 ml of distilled

water. The mixture will be agitated on a rotary shaker for 2 h and then filtered through Whatman filter paper no. 1. The supernatant was used for the estimation of the citric acid by acetic anhydride and pyridine method of Marier and Boulet.

Estimation

After incubation, the filtrate and mycelia are separated using Whatman's filter paper. About 50 ml of filtrate is collected and treated with 5 ml $CaCo_3$ forming calcium citrate precipitate. The contents are filtered, and the residue is collected, and it is treated with equal quantity of concentrated H_2SO_4. It is washed with distilled water. Filtrate containing citric acid is subjected to quantitative test. About 1 ml of pyridine and 5 ml of acetic anhydride added. Incubate for 30 min in ice bath. The standard is prepared by different volumes of citric acid (100 mg of citric acid is dissolved in 100 ml of DW), i.e., 0.2–1 ml in 5 different test tubes. The volume is made up to 1 ml with DW. 1 ml of pyridine and 5 ml of acetic anhydride are added. The blank is prepared by taking 1ml of DW in the place of citric acid. It is then kept for incubation in ice bath for 30 min. The absorbance is read at 420 nm. Concentration of citric acid is found by plotting graph of absorbance versus concentration.

Titration Method

10 ml of the sample is taken in a conical flask. 0.1 N NaOH is taken in a burette. 2–3 drops of phenolphthalein indicator was added to the sample. The sample was titrated with 0.1 N NaOH until permanent pink color is obtained.[10]

$$\% \text{ citric acid} = \frac{\text{Normality} \times \text{volume of 0.1 N NaOH} \times \text{equivalent weight of citric acid}}{\text{Normality} \times \text{volume of 0.1 N NaOH} \times \text{equivalent weight of citric acid}}$$

Confirmatory Tests

2 ml of test sample + 1 drop dil. NH_4OH+ excess cadmium chloride solution. Boil for 15 min in water bath. Positive test indicated by gelatinous white precipitate.

2ml of test sample + 1 drop dil. NH_4OH+ excess silver nitrate solution boil for 15 min. Positive test indicated by Blackish silver mirror.[11]

High-Performance Liquid Chromatography (HPLC) Analysis

Mobile phase, acetonitrile, and water are used as mobile phase in the ratio 40:60, respectively. Standard preparation: citric acid standards of 1 mg/ml were prepared from citric acid and distilled water. These were then transferred to the test tubes for HPLC analysis. 1ml of the standard was diluted with 9 ml of distilled water and filtered using the microfilters of 0.45 µ pore size. Sample preparation: 1 ml of the sample was diluted with 9 ml of distilled water and it was filtered using the microfilters of 0.45 µ pore size. Instrument preparation: The HPLC column used is C18. The system operated at a flow rate of 0.5 mL/min, the injection volume was 25 µL, and the external temperature control column oven was set at 35°C. The detection wavelength was 214 nm. Procedure: The instrument is kept ready for the analysis. 25 µL of the standard is injected and it is kept for 20 min run time. The retention time of the standard is noted on the chromatogram. Then, 25 µL of the sample is injected and the retention time of the standard is compared. Concentration of the citric acid present in the sample is found out.[9]

RESULTS AND DISCUSSION

Spore Count

One disc was smeared with *A. niger* and dipped in distilled water and mixed properly.

1 ml of this sample was taken and spore count is done using hemocytometer [Figures 1-3].

$$\text{Spores/ml} = \frac{A+B+C+D}{4} \times 10^4$$

$$= \frac{67 + 51 + 54 + 48 * 10^4}{4}$$

$=55\times10^4$ spores/ml

4 discs = $(55\times10^4)\times4 = 220\times10^4$ spores/ml

After the 6 days of incubation good growth was observed both in solid and suspension state fermentation. To obtain in the solution

Figure 1: (a and b) Growth in solid and suspension states

form for solid state the substrate was dissolved in 100 ml distilled water. The substrates were filtered twice, first with the strainer and then with Whatman filter paper to obtain a pure solution. The pH was checked for all the solutions using the pH strips. 10 g, 12.5 g, and 15 g for both solid and suspension state fermentation showed acidic pH whereas the rest showed neutral pH.

Confirmatory Test for Citric Acid: Calcium Chloride Test

Positive result was seen with the formation of white precipitate which confirms the presence of citric acid [Figure 4].

Silver Nitrate Test

Positive result was seen with the formation of blackish silver ring which confirms the presence of citric acid [Figure 5].

Figure 2: Filtration of sample

Figure 3: pH determination

Standard citric acid sample was prepared taking 100 mg of citric acid crystals and dissolving it in 100 ml distilled water. Estimation was done to find the standard values. The graph was plotted in Sigma plot taking concentration in X-axis and OD at 420 nm in Y-axis. Straight line passing the origin is obtained giving the slope value as 0.057. From the equation y = mx where y is the OD at 420 nm, x is the concentration of the unknown sample (mg/ml), and m is the slope. A standard graph was plotted to obtain the concentration of the unknown sample.

From the standard graph and equation y = mx, we obtained the concentration for the SSF substrates. From 5 g to 12.5 g, there was an increase in the concentration of citric acid whereas it dropped down at 15 g. Highest concentration 4.74 mg of citric acid at 0.25 OD was observed at 12.5 g of the substrate. From the graph plotted in sigma plot taking weight of the substrate in X-axis and concentration of citric acid in Y-axis, we obtain the optimum weight for the production of citric acid which was found to be 12.5 g.

From the standard graph and equation y = mx, we obtained the concentration for the suspension state fermentation substrates. From 5 g to 12.5 g, there was an increase in the concentration of

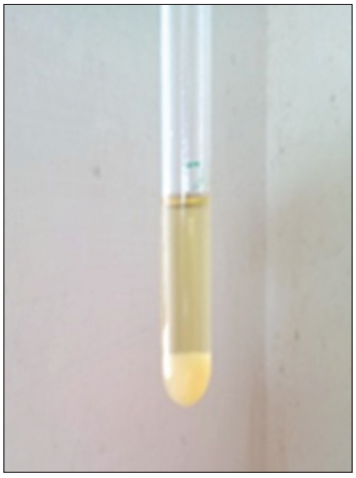

Figure 4: Calcium chloride test

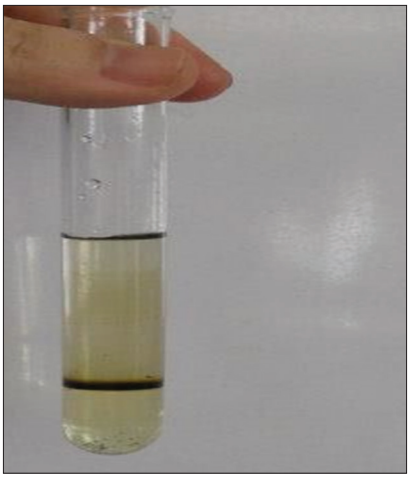

Figure 5: Silver nitrate test

Table 1: Estimation of Standard

Citric acid (ml)	Concentration of citric acid (mg/ml)	DW (ml)	Acetic anhydride (ml)	Pyridine (ml)	Incubate in ice bath for 30 min	OD at 420 nm
0	0	1	5	1		0
0.2	2	0.8	5	1		0.02
0.4	4	0.6	5	1		0.03
0.6	6	0.4	5	1		0.03
0.8	8	0.2	5	1		0.04
1	10	0	5	1		0.05

Table 2: Estimation of citric acid in solid state fermented sample

Weight of substrate (g)	Sample (ML)	concentration of H_2SO_4+distilled water	Acetic anhydride (ml)	Pyridine (ml)	Incubate in ice bath for 30 min	OD at 420 nm	Concentration (mg)
5	1		5	1		0.06	1.138
7.5	1		5	1		0.19	3.6
10	1		5	1		0.22	4.17
12.5	1		5	1		0.25	4.74
15	1		5	1		0.16	3.03

Table 3: Estimation of citric acid in suspension state fermented sample

Weight of substrate (g)	Sample (ml)	Concentration of H_2SO_4+Distilled water	Acetic anhydride (ml)	Pyridine (ml)	Incubate in ice bath for 30 min	OD at 420 nm	Concentration (mg/100 ml)
5	1		5	1		0.02	0.3
7.5	1		5	1		0.03	0.5
10	1		5	1		0.28	5.3
12.5	1		5	1		0.51	9.6
15	1		5	1		0.17	3.2

citric acid whereas it dropped down at 15 g. Highest concentration 9.6 mg/100 ml of citric acid at 0.51 OD was observed in 12.5 g of the substrate. From the graph plotted in sigma plot taking weight of the substrate in X-axis and concentration of citric acid in Y-axis, we obtain the optimum weight for the production of citric acid which was found to be 12.5 g.

Estimation of Citric Acid by Titration Method

Change of colorless to permanent pink color showed the presence of citric acid [Figure 4-6].

$$\% \text{ Citric acid} = \frac{\text{Normality} \times \text{volume of 0.1 N NaOH} \times \text{equivalent weight of citric acid}}{\text{weight of the sample} \times 10}$$

Normality of the sample solution
Equivalent weight of the citric acid - 24
Weight of the sample −9.8 g

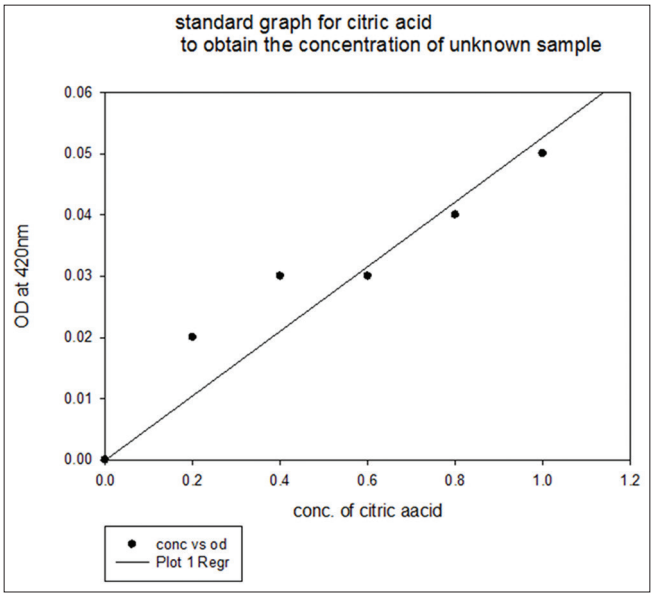

Figure 6: Standard graph for citric acid

Table 4: Estimation by titration method in solid-state fermented sample

Weight of the substrate (g)	Initial, V_1 (ml)	Final, V_1 (ml)	V_2–V_1 (ml)	% Citric acid
5	0	0.5	0.5	0.061
7.5	0.5	1.1	0.6	0.088
10	1.1	1.5	0.4	0.039
12.5	1.5	1	1	0.24
15	3.5	4.0	0.66	0.088

Table 5: Estimation by titration method in Suspension State fermented sample

Weight of the substrate (g)	Initial, V_1 (ml)	Final, V_1 (ml)	V_2–V_1 (ml)	% Citric acid
5	0	1.2	1.2	0.35
7.5	1.2	2	0.8	0.075
10	2	4.8	2.8	1.92
12.5	4.8	10	5.2	6.62
15	10	11.2	1.2	0.35

Table 6: Estimation of citric acid in the samples by varying inoculum size

Number of discs	Sample (ml)	Concentration of H_2SO_4+distilled water	Acetic anhydride (ml)	Pyridine (ml)	Incubate in ice bath for 30 min	OD at 420 nm	Concentration (mg/ml)
2	1		5	1		0.1	1.89
3	1		5	1		0.2	3.79
4	1		5	1		0.49	9.29
5	1		5	1		0.55	10.43
6	1		5	1		0.4	7.59

Table 7: Estimation by titration method in suspension state fermented sample for varying inoculum size

Number of discs	Initial, V_1 (ml)	Final, V_1 (ml)	V_2–V_1 (ml)	% Citric acid
2	0	4.5	4.5	4.9
3	4.5	8	3.5	3
4	8	12	5	6
5	14.5	21.4	6.9	11.6
6	21.4	24.5	3.1	2.3

Percentage of citric acid in SSF is 0.24 whereas in a suspension state fermentation is 6.62 for 12.5 g weight of the substrate [Tables 1-7]. Volume obtained in suspension state was more compared to SSF.

From the standard graph and equation y = mx, we obtained the concentration for the varying inoculum size in 12.5 g of substrate by suspension state fermentation. From 2 to 5 discs, there was increase in the concentration of citric acid whereas it dropped down at the 6th disc. Highest concentration 10.43 mg/100 ml of citric acid at 0.55 OD was observed when 5 discs were added to the 12.5 g of substrate. From the graph plotted in sigma plot taking number of discs in X-axis and concentration of citric acid in Y-axis, we obtain the optimum inoculum size for the production of citric acid which was found to be 275*10^4 spores/ml by the addition of 5 discs.

Volumetric analysis of the samples was done by titration method. Percentage of citric acid was found to be 11.6 by the addition of 5 discs which was the highest compared to all.

HPLC analysis was done to confirm the presence of citric acid by comparing the retention time of standard and the sample solution. The standard showed peaks at retention time 2.508 min and 4.07 min. The sample of optimum weight 12.5 g obtained by suspension state fermentation showed two peaks at retention time 2.531 min and 4.1 min. As the retention time of both the standard and sample solution matches closely it confirms the presence of citric acid in our study . This confirms the presence of citric acid.

The comparative study of both solid and suspension state fermentation is shown in Figure 4. There was an increase

seen from 5 g to 12.5 g weight of the substrate in both solid and suspension state fermentation followed by decrease in the production by 15 g weight of the substrate. Highest concentration of 9.6 mg/ml citric acid was obtained from suspension state fermentation with percentage citric acid found to be 6.62%. Thus, further experiments were carried out by suspension state fermentation. In comparison to our study results following findings found. Okara (soy-residue), a cellulosic byproduct of the soymilk and tofu industry, was used for the production of citric acid by SSF using a cellulolytic *A. terreus* and citric acid producing *A. niger*. Okara supplemented with ammonium sulfate (0·10% N) when fermented by *A. niger* with simultaneous saccharification using *A. terreus* at pH 8·3 and incubation temperature of 30°C resulted in the production of 5·10 g citric acid/100 g dry solids by the 11th day.[3] A SSF process is described for the production of citric acid from apple pomaceby *A. niger* NRRL 567. The yields of citric acid varied with the pomace varieties and were dependent on (1) the amount of methanol present in the pomace, and (2) the fermentation time and temperature. The process yielded as much as 90 g citric acid/kg apple pomace fermented in the presence of 3–4% methanol at 30°C in 5 days.[4] Glycerol and glycerol-containing wastes from biodiesel manufactures carbon energy source for microbial production of citric acid. *Yarrowia lipolytica* N15 produces citric acid. Pure glycerol produce 98 g/l of citric acid whereas glycerol containing biodiesel wastes produce 71 g/l of citric acid by suspension state fermentation.[6]

Citric acid production from different agronomic waste such as grapes, orange, apple, vegetable, tapioca, and coconut husk was carried out using *A. niger* isolated from decayed fruit. Citric acid production was performed by SSF and estimated on 5th, 7th, and 9th days of fermentation. Highest level of citric acid production was noticed on 9th day of fermentation by all the six substrates than the 7th and 5th days of fermentation. Among the six substrates used higher levels of citric acid production were noticed against decayed fruit waste such as grape, orange, and apple but it was low with vegetable, tapioca, and coconut husk.[7]

Citric acid was quantified using HPLC. Eight orange juice samples were analyzed ranging in concentration from 36 to 427 mg/ml of citric acid. California navel orange had the highest citric acid concentration followed by 100% orange juices, flavored orange juices, and lowest concentration in Nestle's juicy juices, the 100% mixed fruit juice [Figure 7-12].[8]

CONCLUSION

Suspension state fermentation method gives higher yield of citric acid using oil seed cake as the substrate from *A. niger* than SSF method. The optimum weight was found out to be 12.5 g both in solid and suspension state fermentation

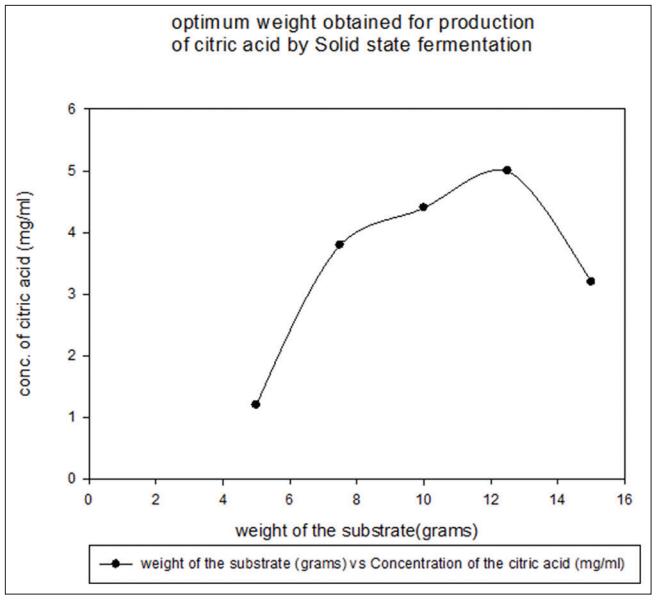

Figure 7: Optimum weight for production of citric acid by solid state fermented sample

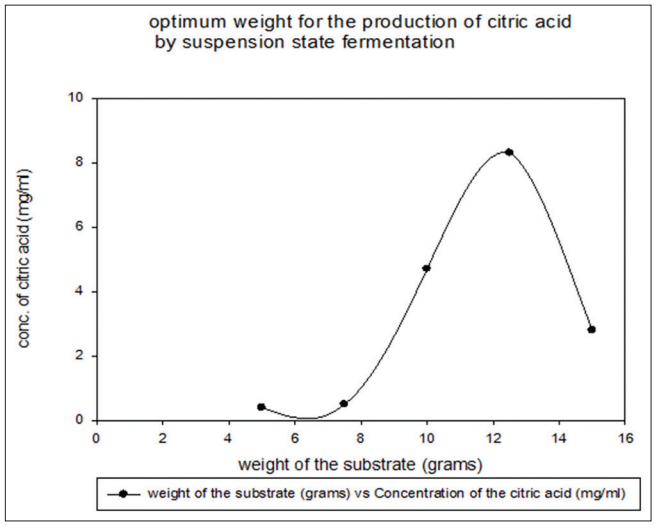

Figure 8: Optimum weight for production of citric acid by suspension state fermented sample

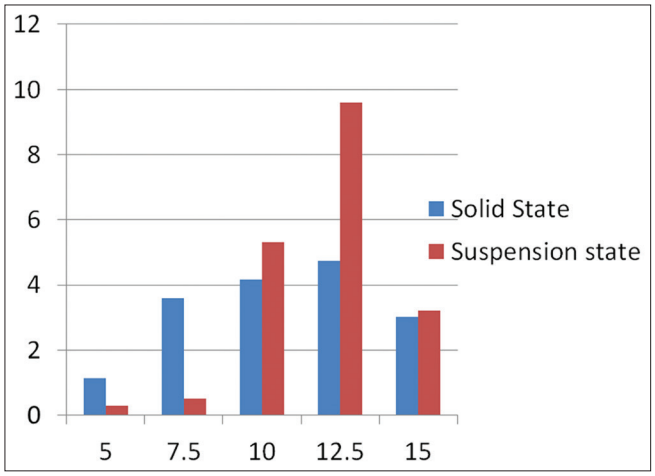

Figure 9: Comparison of citric acid production in solid and suspension state fermentation

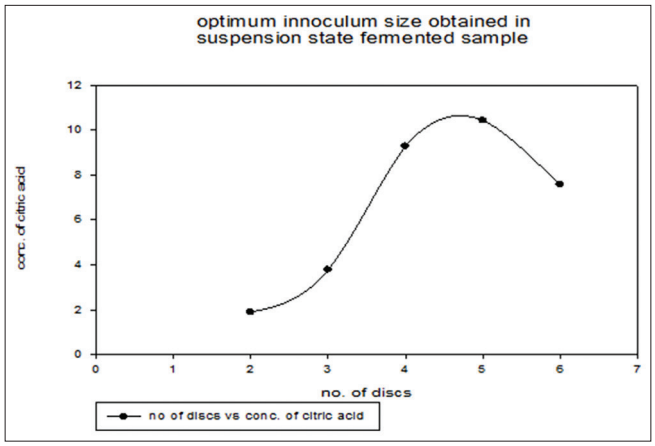

Figure 10: Optimum inoculum size obtained

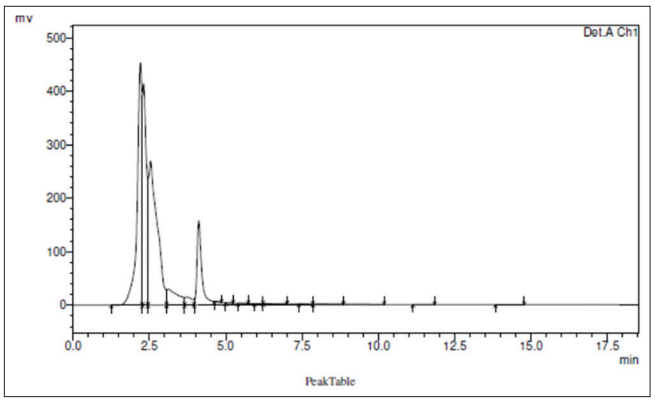

Figure 11: High-performance liquid chromatography citric acid standard

method. The optimum inoculum size was found out to be 275*10^4 spores/ml by suspension state fermentation method. The highest yield of 10.43 mg/ml of citric acid was obtained by taking substrate weight 12.5 g and inoculum size 275*10^4 spores/ml by suspension state fermentation method.

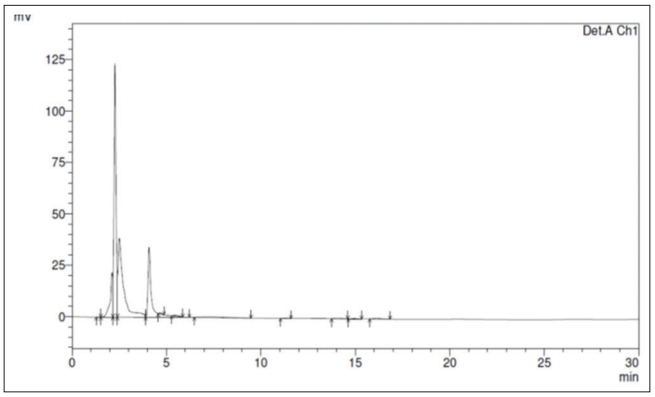

Figure 12: High-performance liquid chromatography analysis for citric acid sample of 12.5 g

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