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Production of citric acid by *Yarrowia lipolytica* using biodiesel derived glycerol

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Crude glycerol, a by product of Jatropha biodiesel was used as carbon source for production of citric acid by *Yarrowia lipolytica* MTCC 35. Concentrations of crude glycerol ranging from 1 to 4 per cent were taken in buffered and unbuffered broth for the optimized production of citric acid by *Yarrowia lipolytica* MTCC 35. The amount of citric acid produced in buffered broth (11.2 gl^{-1}) was doubled when compared to unbufferd broth (5.2 gl^{-1}). Quantitative estimation through HPLC also confirmed the same.

Key words : Citric acid, *Yarrowia lipolytica*, Crude glycerol-biodiesel by product

INTRODUCTION

Citric acid is the most important organic acid produced in tonnage by fermentation. Global production of citric acid in 2004 was about 1.4 million tones (Soccol *et al*., 2003.) Citric acid is widely used to impart a pleasant, tart flavour to foods and beverages. It also finds applications as a function of additive detergents, pharmaceuticals, cosmetics and toiletries. Raw glycerol is an important feed stock when bio-diesel is applied on a large scale production. With the production of 10 kg of biodiesel from rape seed oil, one kg of glycerol becomes available. Numerous carbon sources, such as molasses, n-paraffins, hexadecanes, edible oils, glucose, starch hydrolysates etc., have been used to produce citric acid from *Yarrowia lipolytica*. Although glycerol has been widely used as a carbon source in the production of 1,3 propane diol. (Biebl and Marten, 1995). The aim of present study was to investigates utilization of crude glycerol by Yarriowia lipolytica for citric acid production.

MATERIALS AND METHODS

The standard strain, *Yarrowia lipolytica* was obtained from Microbial type culture collection (MTCC 35), IMTECH, Chandigarh. The Raw material of crude glycerol, a by product of Jatropha biodiesel, obtained from bio energy department, Tamil Nadu Agricultural University (TNAU), India.

Production of citric acid on crude glycerol:

To test the ability of *Yarrowia lipolytica* MTCC 35 for citric acid production on crude glycerol, the glucose in the liquid broth (Papanikolaou *et al.*, 2002) was replaced with crude glycerol. This broth was named as unbuffered broth (UBM). The buffered broth (BM) was prepared by increasing the concentration of K_2HPO_4 and Na_2HPO_4 to 12 gl^{-1} individually. The citric acid production was analysed by inoculating loop full cells into 250 ml Erlenmeyer flasks containing 50 ml sterile broth and incubated in a rotary shaker at 28°C with 200 rpm agitation for seven days. After incubation cells were harvested by centrifugation and dried at 80°C until the constant weight was reached. Filtered aliquots were used to determine quantitative estimation of citric acid. The internal standard with glucose as carbon source also maintained to compare the citric acid production.

Determination of citric acid:

Incubated flasks containing yeast cells were removed and harvested by centrifugation. After centrifugation, the filtered aliquots of the culture medium were analysed for the presence of citric acid. 10 ml of the supernatant samples were taken and volume was made up to 20 ml by adding distilled water. Then a few drops of phenolphthalein indicator were added and the material was titrated against 0.1 N NaOH to a light pink colour end point. The per cent acidity was calculated based on the following formula:

$$\text{Per cent acidity} = \frac{\text{Vol. of 1N NaOH consumed}}{\text{Wt. of the sample}} \times 100$$

From the total acidity, citric acid content was

estimated by multiplying conversion factor of (0.7) with per cent acidity.

Citric acid (gl^{-1}) = 0.7 x per cent acidity

Effect of different concentrations of crude glycerol for maximum production of citric acid In order to optimize the crude glycerol concentration for maximum citric acid production, Buffered (BM) and Unbufferd (UBM) broth as mentioned earlier was prepared with different concentration of crude glycerol (1, 2, 3, and 4 per cent) taken in a 250 ml Erlenmeyer flasks containing 50 ml of the medium, sterilized and inoculated with loop full of *Yarrowia lipolytica* 35, and incubated in an rotary shaker at 28°C with 185 rpm agitation for seven days. After incubation cells were harvested by centrifugation and dried at 80°C until the constant weight was reached. Filtered aliquots were used to determine quantitative estimation of citric acid as described earlier (3.10.2).

Determination of citric acid by HPLC:

Filtered aliquots were taken for the determination of citric acid by using high performance liquid chromatography (HPLC) as described by Papanikolaou *et al.* (2002). Sample was prepared by centrifuging the culture broth at 10,000 rpm for 10 min in a centrifuge supernatants were taken and filtered through 0.22 µm cellulose - acetate membrane before direct injection in HPLC. Standard citric acid was purchased from commercial source (Sigma, Milano, Italy). 1 mg of standard citric acid was diluted in 1 ml of Milli - Q water; the sample was passed through 0.22 µm cellulose acetate membrane before injection in HPLC. The instrument used was Shimadzu HPLC system with LC - 8A preparative liquid chromatography pump, with UV detector. Citric acid was determined by C18 column at a wave length of 210 nm, flow rate used in this experiment was 1.0 ml. 25 µl of standard and sample were injected. Sample citric acid was identified with the retention time of standard, and concentration was calculated by comparing peak area by using following formula.

$$\text{Amount of Citric acid (µg/g)} = \frac{AS}{Astd} \times \frac{M}{M1} \times \frac{V}{V1} \times F$$

AS - Sample area; Astd - Standard area; M- µl of standard injected (25 µl);

M_1- weight of the sample (g); V- Volume of the final extract (ml) 1.5 ml;

V_1- µl of sample injected; F- Recovery factor - 97 per cent

RESULTS AND DISCUSSION

Utilization of crude glycerol, a by product of biodiesel, for the production of citric acid

Crude glycerol, a by product of jatropha oil biodiesel was obtained from Department of Bio energy, TNAU, Coimbatore and used as carbon source for production of citric acid by *Yarrowia lipolytica* MTCC 35 a potential producer of citric acid from crude glycerol obtained from vegetable oil. Hence to evaluate the citric acid production of *Yarrowia lipolytica* MTCC 35 on crude glycerol, standard yeast *Yarrowia lipolytica* MTCC 35 was obtained from IMTECH Chandigarh, India. The citric acid production was compared with glucose as a carbon source.

Glucose was used as carbon source for citric acid production in *Yarrowia lipolytica* MTCC 35. To test the citric acid production of *Yarrowia lipolytica* MTCC 35 on crude glycerol, concentrations ranging from 1 to 4 per cent were taken in buffered and un buffered medium. The result revealed that the citric acid was produced significantly at 3 per cent concentration in both buffered and unbuffered broth. Where as the amount of citric acid produced in buffered broth (11.2 gl^{-1}) was doubled when compared to unbufferd broth (5.2 gl^{-1}). Concentration of crude glycerol above 3 per cent showed decreased amount citric acid in both buffered and unbuffered broth (Table 1).

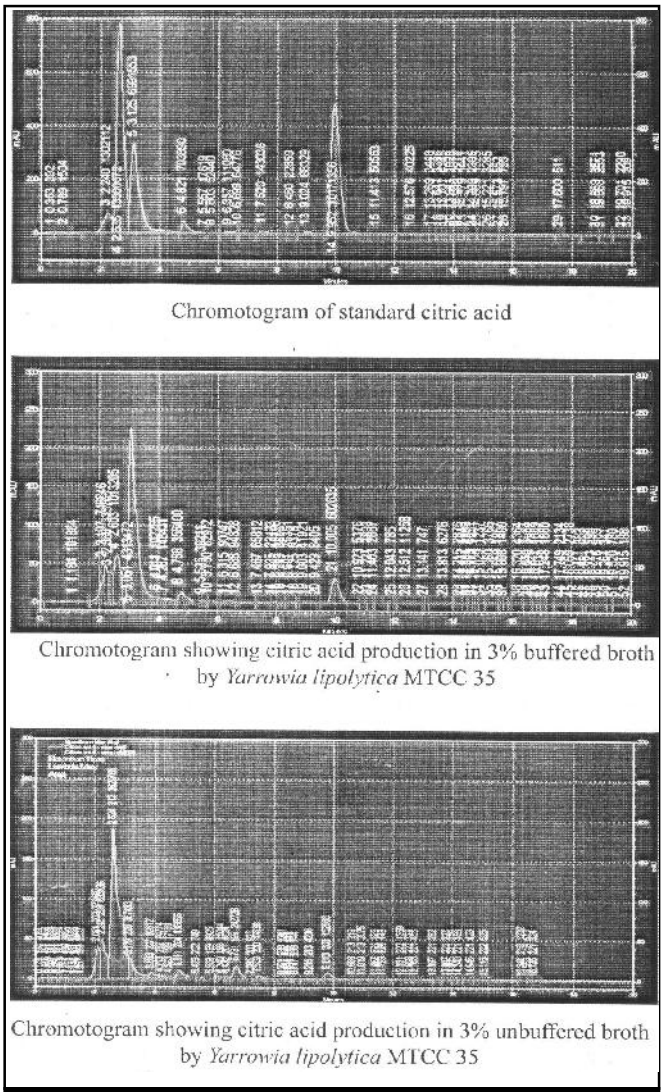
Table 1 : Citric acid production by *Yarrowia lipolytica* MTCC 35 using glycerol as carbon source

Glycerol concentration (%)	Unbuffered broth (UBM)		Buffered broth (BM)	
	Citrate ($g\ l^{-1}$)	Biomass ($g\ l^{-1}$)	Citrate ($g\ l^{-1}$)	Biomass ($g\ l^{-1}$)
1	3.0	3.5	3.5	3.8
2	4.3	3.7	9.3	5.2
3	5.2	3.1	11.2	9.2
4	2.0	2.8	10.1	8.2
S.E. ±	0.506	0.608	0.775	0.763
C.D. (P=0.05)	1.168	1.403	1.788	1.759

Determination of citric acid by HPLC:

HPLC chromatogram of standard culture *Yarrowia lipolytica* MTCC 35 on three per cent buffered medium (BM) showed 5.75 µg/g of citric acid concentration in contrast to three per cent unbuffered medium (UBM) showed 1.45 µg/g of citric acid concentration (Fig. 1).

Raw glycerol is an important feed stock when bio-diesel is applied on a large scale production. With the production of 10 kg of biodiesel from rapeseed oil, 1 kg of glycerol was obtained as byproduct (Meesters *et al.*, 1996). Numerous carbon sources, such as molasses, n-paraffins,



Chromotogram of standard citric acid

Chromotogram showing citric acid production in 3% buffered broth by *Yarrowia lipolytica* MTCC 35

Chromotogram showing citric acid production in 3% unbuffered broth by *Yarrowia lipolytica* MTCC 35

Fig 1. : Determination of citric acid by HPLC

hexadecanes, edibleoils, glucose, starch hydrolysates etc., have been used to produce citric acid from *Yarrowia lipolytica* (Rane and Sims, 1993). Although glycerol has been widely used as a carbon source in the production of 1,3 propane diol (Biebel and Marten, 1995).

In general citric acid is commercially produced by submerged microbial fermentation of molasses. A large number of microorganism including fungi, bacteria and yeasts have been employed for citric acid production. The fermentation process using *Aspergillus niger* is still the main source of citric acid world wide (Soccol *et al.*, 2003). Yeast strain namely *Yarrowia lipolytica* has the ability to utilize raw glycerol as carbon source and produce citric acid in considerable quantities (Papanikolaou *et al.*, 2002)

The standard strain, *Yarrowia lipolytica* MTCC35 was obtained from IMTECH, Chandigarh to study the utilization of crude glycerol obtained from jatropha biodiesel unit to produce the citric acid. Cell growth and citric acid production was determined by using different concentrations of crude glycerol and compared with glucose (2 per cent), because glucose is the major carbon source for citric acid production (Papanikolaou *et al.*, 2002).The biomass yield on glycerol was slightly higher than that obtained on glucose, whereas citric acid production was similar for both substrates used. Papanikolaou *et al.* (2002) studied different concentrations of crude glycerol for its citric acid production. The Study revealed that as much as glycerol concentrations increases citric acid production also increased.

Maximum amount of biomass and citric acid was obtained in buffered when compared to unbuffered broth. The decreased yield in unbufferd broth might be due to an increased energy for maintenance requirement, as well as excessive CO_2 production during culture in unbufferd broth. After the exhaustion of sugars from the culture medium, some organism starts consuming citric acid produced in the medium. Analysis of citric acid through high performance liquid chromatography was studied by Papanikolaou *et al.* (2002), citric acid concentration was estimated by peak area obtained from the commercial standards.

The present study also revealed that biodiesel by product can be alternatively utilized for the production of citric acid by using yeast.

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