Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 6 (6): 568-574, 2011
ISSN 1816-4935 / DOI: 10.3923/jm.2011.568.574

A Comparative Study on Two *Yarrrowia lipolytica* Strains for Optimum Citric Acid Production

Mirbagheri Maryam, Nahvi Iraj and Emtiazi Giti
Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, I.R. Iran

Corresponding Author: Maryam Mirbagheril, Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, PostCode 81746-73696, I.R. Iran Tel: +98-0311-6505644 Fax: +98-0311-7932456

ABSTRACT

The aim of this study was to investigate the influence of various carbon sources like glucose, glycerol, olive oil and different nitrogen sources including yeast extract, tripton, peptone, soybean and $(NH_4)_2SO_4$ with various concentrations on production of citric acid by two strains *Yarrowia lipolytica* DSM3286 and *Yarrowia lipolytica* M7. Present results proved glucose and olive oil as the best carbon sources in media for citric acid production by these strains. Decreasing nitrogen rate is the most important factor influencing citric acid production by yeasts. Decreasing in $(NH_4)_2SO_4$ from 0.5 g L^{-1} to 0.25 g L^{-1} in production media improved production of citric acid in *Y. lipolytica* DSM3286 (43.3 g L^{-1}) and *Y. lipolytica* M7 (55.5 g L^{-1}) to 56.09 g L^{-1} and 65.12 g L^{-1} alternatively. Also soybean (0.5 g L^{-1}) for *Y. lipolytica* M7 and yeast extract (0.5 g L^{-1}) for *Y. lipolytica* DSM3286 were the suitable organic nitrogen sources in production media. This study characterized citric acid accumulation is a very complex process during which various metabolic and morphological changes take place in a complex form.

Key words: Citric acid, *Yarrowia lipolytica,* carbon source, nitrogen source, optimization

INTRODUCTION

Citric acid is a chemical, which is produced solely by a biotechnological process. It is widely used in a great number of applications, ranging from the food processing to the pharmaceutical industry and to other areas of the chemical industry like cosmetics and applied as a buffering and chelating agent (Sanz *et al*., 2004; Soccol *et al*., 2006). Citric acid production in all developed countries follows a conventional procedure, which involves the use of *Aspergillus niger* (as producer) and molasses (as substrate) (Mazhar *et al*., 2003; Xie and West, 2006). Using the yeasts like *Yarrowia lipolytica* would have several advantages compared to the *Aspergillus*, including a smaller sensitivity to low dissolved oxygen concentrations and heavy metals and higher product yield, less waste, slighter toxicity, smaller susceptibility of the yeast cells against mechanic shear forces and the utilization of various raw materials as substrates. Wild type strains of these yeasts can use a wide spectrum of carbon sources as substrates for overproduction of organic acids. Therefore yeasts are good candidate for citric acid production instead of *A. niger* (Vandenberghe *et al*., 1999; Babu and Rao, 2007b; Lodhi *et al*., 2001).

Different aspects of formation of citric acid by yeasts using various C-sources have been investigated over the past 35 years. The effects of different growth culture compositions and other parameters have been simultaneously studied (Treichel *et al*., 2009; Rane and Sims, 1993;

Babu and Rao, 2007a). Yigitoglu, (1992) and Soccol *et al.* (2006) represented physical condition like pH, temperature and aeration could affect citric acid production by yeast (Yigitoglu, 1992). The influence of air saturation and temperature on continuous citric acid secretion was studied in chemostat cultures of *Candida oleophila* ATCC 20177 by Anastassiadis and Rehm (2006).

Citric acid production by *Candida* strains under intracellular nitrogen limitation and effect of nitrogen and biomass concentration on yield and productivity studied in other research. In the literature, a comparison of different strains has been carried out by Kamzolova *et al.* (2005) and Treichel *et al.* (2009).

In this study, two wild type strains of yeasts including *Y. lipolytica* DSM3286 and *Y. lipolytica* M7 selected. We investigated optimization of the citric acid production by changing in composition of production media like using different carbon and nitrogen sources and changes in their concentrations in base production media then studied optimum initial pH for citric acid production by yeast *Yarrowia lipolytica*.

MATERIALS AND METHODS

Strains and growth conditions: In present study that was done along year 2010, *Yarrowia lipolytica* DSM3286 was obtained from the culture collection of the Germany and *Yarrowia lipolytica* M7 isolated from poultry meat in Microbiology lab of the University of Isfahan. Initially cells were grown aerobically at 29°C in liquid media YPD (yeast extract peptone dextrose medium) about 24 h then transmitted in production media (Lopandic *et al.*, 2006).

Production media and analysis procedures: The composition of fermentation medium for citric acid production was achieved from Papanikolaou *et al.* (2002). Initial pH of the media was adjusted to 7. For cultivation, the strains were grown at 29°C and 200 rpm in 250 mL flasks. All of materials obtained from Merk Company. Assay of citric acid was performed using a K-CITR enzymatic test kit (Megazyme, Wicklow, Ireland) according to manufacturer's recommendations. Optical density (OD660 nm) and dry biomass (filter method) were measured as described in Anastassiadis *et al.* (2002).

Base production medium including 10% glucose; to further study other glucose concentrations like 5, 15 and 20% were investigated. Optimization in kind of carbon sources obtained by using of glycerol and olive oil, *Yarrowia* by lipolytic ability could have useful to wastewater treatment in oil manufactories.

Nitrogen source of base production media ($(NH_4)_2SO_4$) decreased from 0.5 to 0.4, 0.3, 0.25 and 0.1. Studies in kind of nitrogen source changes done by using of peptone, tripton and soybean.

In citric acid production by *Aspergillus niger* usually initial pH adjusted on 3-4. For determination of optimum initial pH to citric acid production by yeast *Y. lipolitica* were changed pH to 3.5, 4.5, 5.5 and 6.5.

Statistical analyses for comparison of results obtained by analysis of variance (one way ANOVA) in SPSS software. The interaction effect of carbon and nitrogen sources was found to be significant ($p<0.05$).

RESULTS

Citric acid production by yeast strains: Yeast strains were cultured on citric acid fermentation medium for 192 hours and quantitative assay of citric acid was determined each 24 h. Results showed that the maximum citric acid production obtained 144 h after inoculation. Two strains of

Yarrrowia lipolytica that use in this study were selected in previous research in Microbiology lab of Isfahan. Wild type *Y. lipolytica* M7 that screened from poultry was produced maximum 55.5 g L^{-1} of citric acid and standard strain *Y. lipolytica* DSM3286 produce citric acid about 43.3 g L^{-1} along 144 h (Fig. 2).

Effect of carbon sources: Study of various glucose concentration proved 5% glucose by significant level ($p<0.05$) is the best for DSM3286 while 10% is the best for M7 and it depends to type of strains. Production of citric acid decreased in high concentration of glucose in batch culture because high osmotic shock (Fig. 1). By replacement of glucose with glycerol and olive oil determined in presence of olive oil, production of citric acid changed to 62.03+ g L^{-1} for M7 and 35.5 g L^{-1} for DSM3286, glycerol have negative effect on citric acid production in compared glucose (Fig. 2).

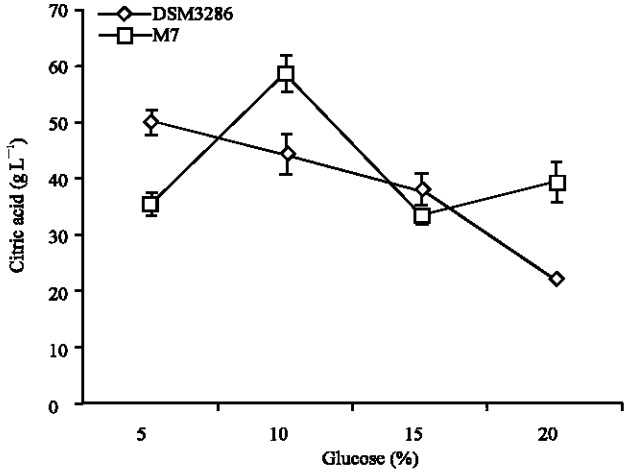

Fig. 1: Citric acid production in different concentrations of glucose by *Y. lipolytica* M7 and *Y. lipolytica* DSM3286

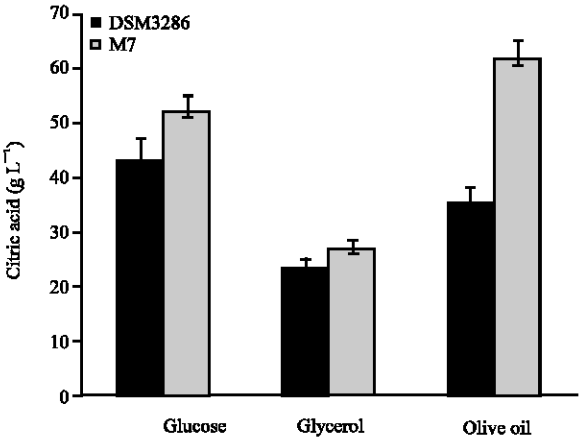

Fig. 2: Effect of various carbon sources (10%) in citric acid production by *Y. lipolytica* M7 and *Y. lipolytica* DSM3286. The interaction effect of carbon sources was found to be significant ($p \leq 0.05$)

Effect of nitrogen sources: Decrease in nitrogen source of production media occurred remarkable increasing of citric acid production by two strains along 144 h in shake flask condition (Fig. 3). Attempt to use 0.25 g L^{-1} $(NH_4)_2SO_4$ increased production of citric acid to 65.12±0.1 for M7 and 56.09±0.1 g L^{-1} for DSM3286. Changes in type of organic nitrogen source in production media determined yeast extract is the best nitrogen source for production of citric acid in DSM3286 and soybean is better for M7 strain (Fig. 4).

Effect of pH: Investigation to determination of optimum initial pH for citric acid production by yeast *Yarrowia lipolytica* showed pH about 6.5 is the best for two strains by significant level ($p<0.05$) compared with other pH (Fig. 5).

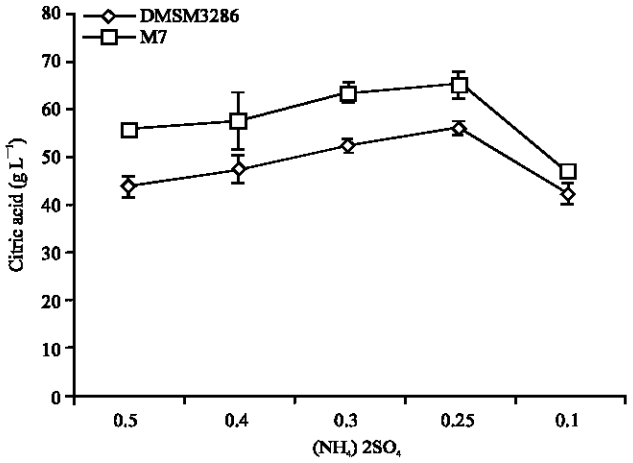

Fig. 3: Study of different $(NH_4)_2SO_4$ in citric acid production by *Y. lipolytica* M7 and *Y. lipolytica* DSM3286 in 144 h. The interaction effect between 0 .5 g L^{-1} and .0.25g L^{-1} was significant ($p \leq 0.05$)

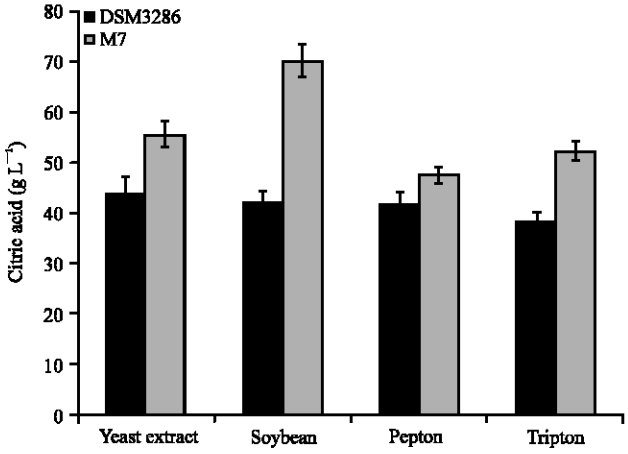

Fig. 4: Effect of various nitrogen sources (0.5 g L^{-1}) on citric acid production by *Y. lipolytica* M7 and *Y. lipolytica* DSM3286. The interaction effect of nitrogen sources were found to be significant ($p \leq 0.05$)

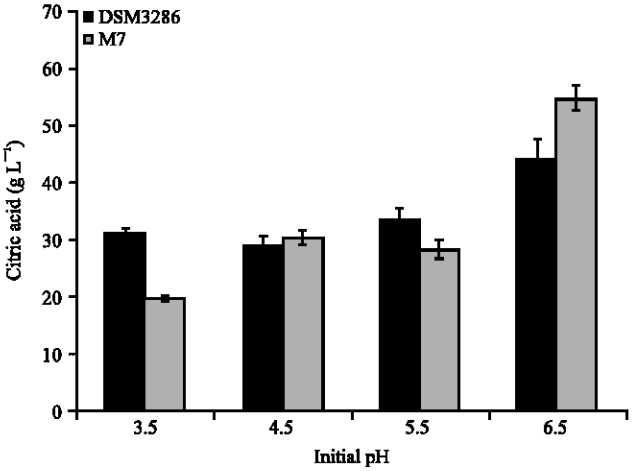

Fig. 5: Effect of changing initial pH on citric acid production by *Y. lipolytica* M7 and *Y. lipolytica* DSM3286 in 144 h

DISCUSSION

In this study attempt to identify a suitable yeast strain and important factors influencing citric acid formation in batch cultures for development of a new process for the production of citric acid in continuous fermentation in future.

Papanikolaou *et al.* (2006) investigated the effect of various concentrations of commercial glucose used as sole substrate on citric acid production by *Y. lipolytica* ACA-DC 50109. They extract 10.5, 15, 20 and 42.9 g L^{-1} citric acid by using of 34, 42, 52 and 149 g L^{-1} glucose in bath culture that their result are in line with present study. Behrens *et al.* (1987) known that production of citric acid takes place when reproductive growth ceases, which is usually caused by exhaustion of nitrogen in the fermentation medium. Barth and Gaillardin (1997) and Antonucci *et al.* (2001) represented *Yarrowia lipolytica* and other Candida strains are able to produce citric acid from various substrates, whereby glucose has generated increasing interest. Investigations of this study were in support with these researchers.

Anastassiadis (1994) showed *Candida oleophila* was identified as the best citric acid producer among several *Candida* strains screened. Final citric acid concentrations achieved by *C. oleophila* of 50.1 g L^{-1} in batch cultures. Their finding similar to our results showed that citric acid formation in various yeasts increased under limitation of nitrogen conditions.

Ammonium nitrogen has been found to be limiting substrate for citrate accumulation by *Y. lipolytica* that influences growth rate, as also reported by Aiba and Matsuoka (1979). A balance between nitrogen concentration and other nutrients is also essential for optimum citrate excretion in yeasts. The importance of nitrogen limitation became clearer upon elemental analysis of biomass composition. Significant differences were found in the intracellular biomass composition of yeast cells from the growth (trophophase) and production phase (idiophase) showed by some studies. While the carbon and hydrogen content showed small changes, the nitrogen content in the cell decreased dramatically in the idiophase. A similar decrease in nitrogen content of *Saccharomycopsis lipolytica* D1805 from 8.5% in the trophophase down to 4% at the end of the exponential phase has been reported by Briffaud and Engasser (1979). Moresi *et al.* (1980) reported a reduction in the intracellular nitrogen fraction from 7-8% to 2.3-4.4% in *Y. lipolytica* ATCC 20346 along growth.

Nitrogen exhaustion in the medium followed by the decrease of intracellular nitrogen content, as well as the rising intracellular levels of NH_4^+ and energy induce a specific active transport system for citrate secretion.

Behrens *et al.* (1987) assumed that the decrease in enzyme activity of various enzymes is responsible for activity loss in citrate production. Lozinov *et al.* (1974) reported an increase in biomass glycogen (15-35%) and lipids (5-20%) during nitrogen limitation in *Candida guilliermondii*. Similarly, *A. niger* has been reported to accumulate carbon in the cell under nitrogen limitation (Kristiansen and Sinclair, 1978). Kozlova *et al.* (1981) found a strong reduction in protein content from 43% in the exponential phase to 17% during the stationary growth phase in *Y. lipolytica*.

The effects of pH and temperature on cell growth and production of citric acid from glucose by *Y. lipolytica* H222 were investigated by Moeller *et al.* (2007). They showed highest concentration of citric acid during the production phase was obtained at 30°C in pH 6 (41 g L^{-1} CA) in bath culture that confirmed our results about pH and temperature.

CONCLUSIONS

In conclusion, by other findings and results of this present work characterized citric acid accumulation is a very complex process during which various metabolic and morphological changes take place in a complex form. According to elemental analysis and fermentation data, nitrogen content in the cells following its extracellular exhaustion is the major factor influencing specific activities in citric acid formation by yeasts. In batch cultures *Y. lipolytica* increasing nitrogen limitation gradually turns yeast metabolism from cellular growth to product formation, until growth almost ceases. This fact explains the negative correlation between growth and production rate.

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