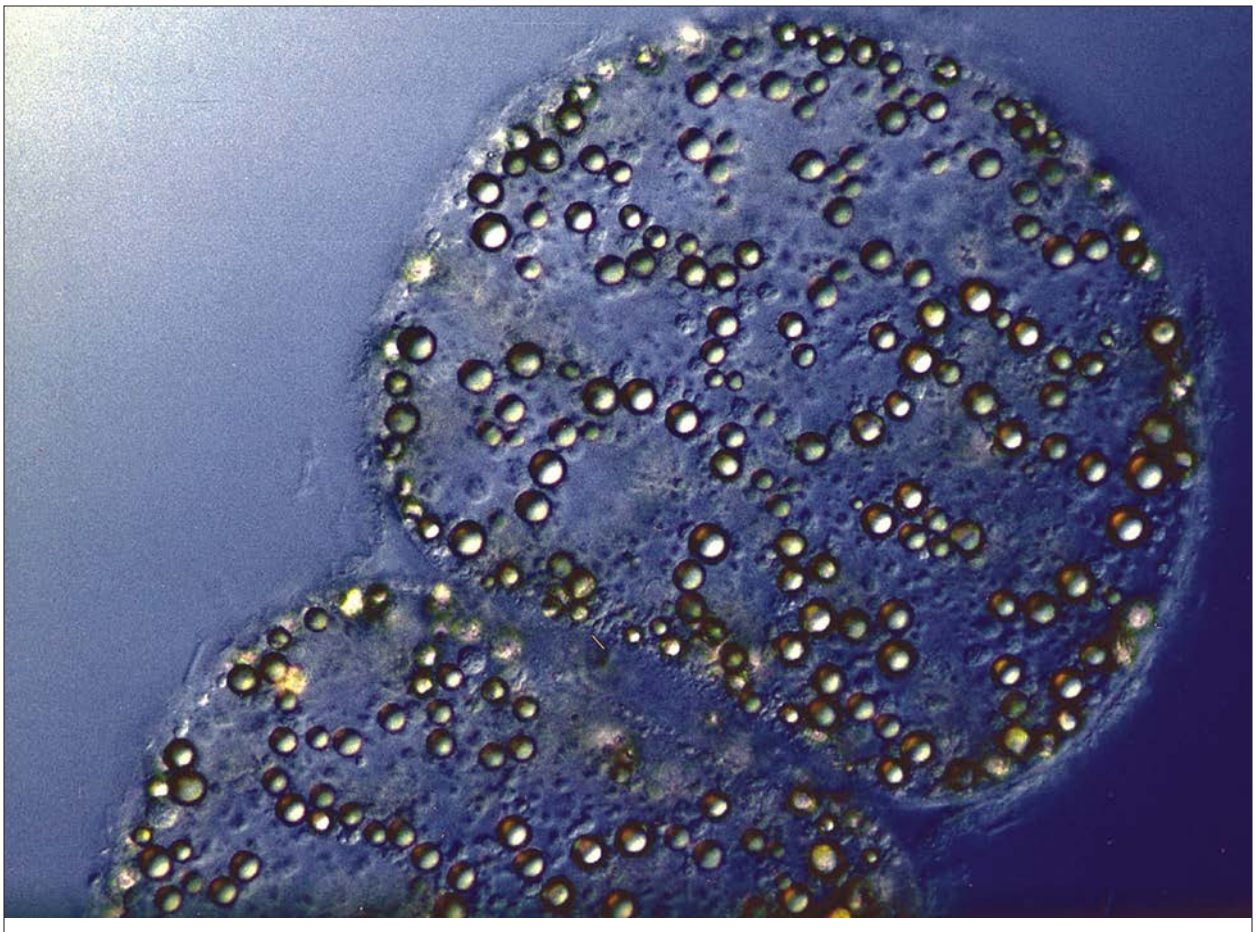

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The Effect of Viscous Substances on Citric Acid Production by *Yarrowia lipolytic*

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ABSTRACT

The viscosity of the medium is one of the important factors for citric acid productivity in shake culture, so in this study the effect of various viscous substances on citric acid production by *Yarrowia lipolytica* DSM3286 and isolated wild type *Yarrowia lipolytica* M7 were investigated. The addition of 0.25 g L^{-1} agar as a viscous additive to the medium containing glucose as a carbon source increased slightly the medium viscosity but substantially increased the citric acid yield on shake culture in *Y. lipolytica* M7 and *Y. lipolytica* DSM3286 by 28 and 40%, respectively. Different morphologies of the DSM3286 strain were observed when cultivations were done in shake culture with or without the addition of agar. Also addition of 0.25 g L^{-1} carboxy methyl cellulose (CMC) had positive effect on citric acid production by *Y. lipolytica* strains however addition of gelatin and alginate had negative results. On the other hand PEG 4000 didn't have significant effect on citric acid production. Since *Y. lipolytica* does not utilize these viscous substances, these results suggested that the viscous substances functioned as protectants for the yeasts from physiological stresses due to shaking and as a consequence resulted in a remarkably increased citric acid yield in shake culture.

Key words: Viscous substances, *Yarrowia lipolytica*, Citric acid, yeast, Optimization, Shake culture

INTRODUCTION

Citric acid has a broad use in the household, in the preparation of numerous industrial products and in many industrial areas such as the food, pharmaceutical and chemical industries (Soccol *et al*., 2006). *Yarrowia lipolytica* is one of the best strains of yeasts that degrade renewable low-cost substrate to produce organic acid especially citric acid and have higher product yield. Using the yeasts like *Y. 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 (Mazhar *et al*., 2003; Xie and West, 2006; Babu and Rao, 2007a). Wild type strains of this yeast can use a wide spectrum of carbon sources as substrates for overproduction of organic acids and commonly used for citric acid production. Therefore, yeasts are good candidate for citric acid production instead of *A. niger* (Vandenberghe *et al*., 1999; Babu and Rao, 2007b). Barth and Gaillardin (1997) and Antonucci *et al*. (2001) note that there are some studies to optimization of citric acid production by *Y. lipolytica* and other yeasts. Physiological stresses and physical changes to cell membrane of yeasts caused by different culture environments, might affect citric acid productivity. Michaels and Papoutsakis (1991) showed that some viscous substances are widely and successfully used as a protectant against fluid-mechanical cell damage in plant and animal cell

cultures (Michaels *et al*., 1991). Although in submerged culture, viscosity of the culture broth is considered to be one of the factors related to productivity (Berovic *et al*., 1991), so far there have been no reports on the effects of viscosity on citric acid productivity from the shake flask culture in yeasts.

This objective of this study was to describe the culture conditions that affect citric acid productivity of *Y. lipolytica* DSM3286 and wild type *Y. lipolytica* M7 hyper-producer of citric acid in shake culture.

MATERIALS AND METHODS

Micro organisms: In the present study, that was done along year 2010, *Yarrowia lipolytica* DSM3286 strain was obtained from the culture collection of the DSM (Deutsche Sammlung von Mikroorganismen, German Collection of Microorganisms) and wild type *Y. lipolytica* M7 isolated from poultry meat in Microbiology lab of University of Isfahan. Initially cells were grown aerobically at 29°C in liquid media YPD (yeast extract 1 g L^{-1}, peptone 1 g L^{-1} and dextrose 2 g L^{-1}) about 24 h then transmitted in production media (Lopandic *et al*., 2006).

Citric acid production: The composition of fermentation medium for citric acid production was KH_2PO_4 7 g, Na_2HPO_4 2.5 g, $MgSO_4$ 1.5 g, $CaCl_2$ 0.15 g, $FeCl_2$ 0.15 g, $ZnSO_4$ 0.02 g, $MnSO_4$ 0.06 g $(NH_4)_2SO_4$ 0.5 g, yeast extract 0.5 g, glucose 100 g L^{-1} (Lopandic *et al*., 2006). Initial pH of the media was adjusted to 6. 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 (OD 660 nm) and dry biomass (filter method) were measured as described by Anastassiadis *et al*. (2002).

Viscous substances and additives: The viscous substances used in this investigation were agar (Merk, German), alginate (Merk company), carboxymethylcellulose (CMC, Yoneyama Chemical Co., Japan), gelatin (Kanto Chemical Co., Japan) and polyethylene glycol 4000 (PEG 4000, Wako). After addition of 0.25 g L^{-1} of viscous substance to the culture medium for each experiment, the initial pH of the medium was adjusted to 7.

Statistical analysis: Statistical analyses for comparison of results obtained by analysis of variance (one way ANOVA) in SPSS software. The interaction effect of comparisons was found to be significant ($p \leq 0.05$).

RESULTS

Growth factors, physical conditions and composition of shake flask cultures influenced growth and metabolite production of yeast cells. Peresent study showed 144 h is the best time for accumulation of citric acid in two strains of *Y. lipolytica*. Initially study of citric acid production proved these strains could produce noticeable amount of citric acid (55.5 g L^{-1} by *Y. lipolytica* M7 and 43.3 g L^{-1} by *Y. lipolytica* DSM3286). Treatment with viscous material showed agar (0.25 g L^{-1}) increased production of citric acid to 63 g L^{-1} in *Y. lipolytica* M7 and to 75.2 g L^{-1} in *Y. lipolytica* DSM3286 that maintain in Fig. 1. This differentiation depends on structure of two yeast strains, study of light microscopic showed *Y. lipolytica* DSM3286 is too long and linear and produced mycelium but *Y. lipolytica* M7 had oval shape that showed in Fig. 2. Moreover,

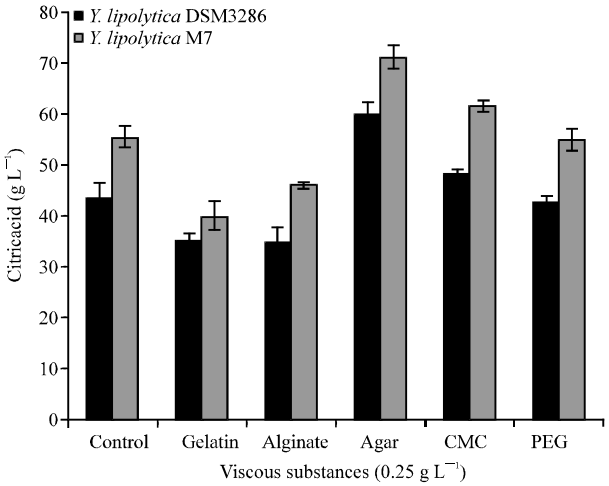

Fig. 1: The effect of various viscose substances on citric acid production by two yeasts *Y. lipolytica* DSM3286 and *Y. lipolytica* M7 along 144 h. Error bars represent the standard error associated with (at least) triplicate experiments

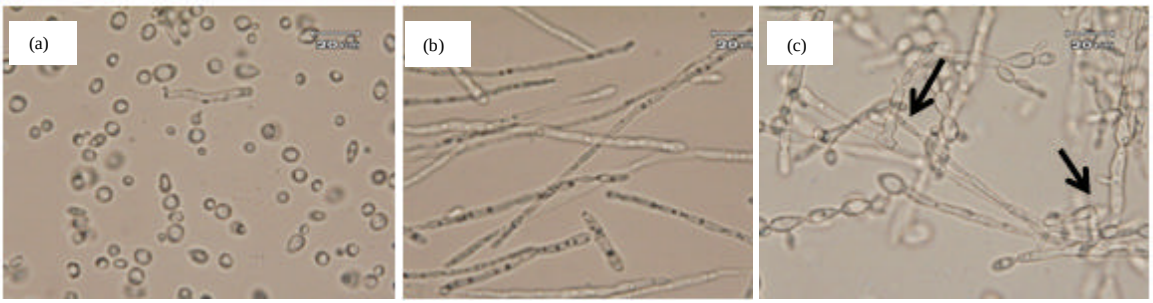

Fig. 2(a-c): Light microscopic structure of two strains of *Y. lipolytica* (a) M7 and (b) DSM3286, yeast cells of DSM3286 is longer and thinner than M7 without treatment with viscous substances. (c) the structure of *Y. lipolytica* DSM3286 in presence of agar as viscous agent after 144 h, the arrows indicate bulbous cells in the mycelia and the scales show 20 μm

differences in mycelial morphology of DSM3286 were also observed after 144 h of cultivation in shake culture with and without the addition of the viscous substances, i.e., agar as shown in Fig. 1, in contrast to in the medium without addition of agar, in the medium with addition of agar, the mycelia were thick with stable spherical aggregates consisting of a denser and branched. Moreover, it was observed that the mycelia in the medium with addition of agar produced more bulbous cells than that in medium without the addition of agar.

Also CMC had positive effect on citric acid production by *Y. lipolytica* strains but gelatin and alginate had negative results. PEG 4000 didn't have significant effect on citric acid production (Fig. 2).

Table 1 determined the yield of citric acid production and changes in dry weight of these strains in absence and presence of variable viscous substances. According to these results maximum yield

Table 1: Comparison between dry weight (g L^{-1}) and yield ($g_{citric\ acid}/g_{carbon\ source}$) in absence and presence of viscous substances along 144 h

	Strains	Control	CMC	PEG	Agar	Alginate	Gelatin
Dry weight (g L^{-1})	*Y. lipolytica* M7	9.32	10.60	11.50	17.83	11.34	12.14
	Y. lipolytica DSM3286	13.70	18.95	14.20	29.33	14.00	13.80
Yield (g_{cit}/g_{glc})	*Y. lipolytica* M7	0.555	0.615	0.549	0.710	0.460	0.367
	Y. lipolytica DSM3286	0.433	0.482	0.427	0.597	0.346	0.35

The interaction effect of this comparison was found to be significant ($p \leq 0.05$)

of citric acid production created by using of agar and minimum yield of production showed by means of gelatin in production medium about two strains. All of viscous substances caused increasing in dry weight but it was more obvious with using of agar in medium especially about *Y. lipolytica* DSM3286.

DISCUSSION

The addition of each viscous substance slightly stimulated yeasts growth. Thus, these results excluded the possibility that the viscous substances could have been used as additional carbon sources for citric acid production or mycelia growth (Behrens *et al*., 1987). Therefore, it seems likely that each viscous substance did not function as a stimulator for growth but rather as a shock-absorbing substance in shake culture. After following the time courses of *Y. lipolytica* DSM3286 in shake culture with or without the addition of the viscous substances, we found that the viscous substances did not affect so much the glucose consumption rate or mycelial growth but substantially stimulated the citric acid productivity of the strain during the entire period of cultivation (data not shown). The physical means of shaking have been reported to cause denaturation of proteins and inactivation of some enzymes as well as the transport of nutrients into cells (Reese, 1980). It is most likely that shear forces cause damage to cell membranes and inhibit microbial growth and productivity (Usami and Taketomi, 1960; Smith *et al*., 1990; Toma *et al*., 1991; Morimura *et al*., 1992). This research confirmed present results. In this study, we demonstrate that culture conditions affect the citric acid productivity of two *Y. lipolytica* strains. Results of Rugsaseel *et al*. (1993) about the effect of viscous material on citric acid production by *A. niger* indicate that physiological stress caused by shaking is one of the important factors that drastically reduced the citric acid productivity. They found that the addition of viscous substances to the medium enhanced the citric acid productivity in shake culture whereas no influence was observed in static cultures that were in support of present results *Y. lipolytica*.

Therefore, we consider that the viscous substances functioned as protectants for the mycelium from physiological stresses due to shaking. Similar phenomena on the effects of some additives as protectants have been demonstrated by other researcher like Kunas and Papoutsakis (1990) who found that the viscosity of the medium affects the structure of turbulence and the transmission of mechanical forces to the cultured animal cells.

According to present results, the addition of viscous substances caused an increase in medium viscosity and thereby in citric acid productivity. On the other hand, the degree of change in the relative viscosity through the addition of different amounts of agar seems to have a negligible effect on the citric acid yield.

Furthermore, we found that the values of the medium viscosity after the addition of each viscous substance and their respective influence on citric acid productivity of *Y. lipolytica* were not correlated with each other. Hence, it is probable that not only the medium viscosity itself but also

some other important factors pertaining to the additives, such as the structure or the molecular mass, affect citric acid productivity in shake culture. These facts are also supported by Rugsaseel *et al.* (1993). Different morphological characteristics were observed in media with and without addition of viscous substances. Interestingly, with the addition of viscous substances, DSM3286 revealed morphological characteristics similar to those found in *A. niger* strains grown in high-citric-acid production medium (Legisa *et al.*, 1981; Ujcova *et al.*, 1980).

CONCLUSION

Results of this study suggest that the protective effects of viscous substances indirectly exerted some biological effects concerning citric acid production. To our knowledge, the present investigation is so far the first examination of the general effects of viscous substances on the stimulation of citric acid production in shake culture of the yeast *Y. lipolytica*. More study is required in order to understand the protective effects of all these viscous substances and the possible mechanisms underlying these effects.

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