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Enhanced Production of Citric Acid in *Yarrowia lipolytica* by Triton X-100

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Abstract Various chemical surfactants could affect permeability of yeast cells. In this study, effects of the surfactant addition upon yeast cells permeability and citric acid (CA) production by *Yarrowia lipolytica* strains DSM 3286 and M7 were investigated. The addition of Triton X-100 increased 1.4–1.8-fold of the maximum CA quantity achieved for both strains, with final CA concentrations ranging between 75–85 g/l that correspond to CA conversion yields per unit of glucose consumed of ~0.80–0.84 g/g. Scanning electron micrographs of yeast cells showed that the cells treated with Triton X-100 had altered cell structure and were smaller and narrower compared with the non-treated ones. The results showed that Triton X-100 could be used in order to increase the efficiency of CA production by *Y. lipolytica* strains.

Keywords *Yarrowia lipolytica* · Citric acid · Triton X-100 · Surfactant · Permeabilization

Introduction

Citric acid (CA) is an important multifunctional organic acid with a broad range of versatile uses in household and industrial applications that has been produced industrially since the beginning of twentieth century. Due to several advantages such as wide substrate spectrum, higher maximal product formation rate, higher product yield, greater tolerance to metal ions and low oxygen concentrations, simple process control, and waste and sewage minimization, the yeast *Yarrowia lipolytica* and *Candida* strains offer

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an alternative to the traditional strain *Aspergillus niger* used for CA production processes [1, 2]. Yeast strains produce CA under intracellular nitrogen limitation [3]. Recently, glycerol as waste, derived from biodiesel manufacture and hydrophobic substrates (e.g., *n*-alkanes, fatty acids, fats, and oils) have been considered as potential substrate for CA production by *Y. lipolytica* [4–9].

Various chemicals can interfere with cell membranes in a way to increase cell permeability to low-molecular-weight solutes. Permeabilization is possible if the chemicals with surfactant property can penetrate the cell wall and reach the membrane. The success of the permeabilization process depends on the composition of the cell wall and the cell membrane [10, 11]. A number of the chemicals or their mixtures have been reported as permeabilization agents for microorganisms such as mixtures of toluene and ethanol [12], various alcohols [13], cetyltrimethylammonium bromide [14], steroidal glycoside, and digitonin [15, 16].

The treated yeast cells by Triton X-100 are able to convert phosphorylate nucleotide monophosphate to nucleotide triphosphate and then, to a final nucleotide derivative (CDP-choline). Untreated cells were not able to carry out these reactions. Probably, the reason is that some changes must occur in the yeast cell surface. Different concentrations of the detergents have a broad range of effects—from increase the permeability of the cell until cell membrane lyses and death of cell—on yeast cells [17].

In this research, we study CA production in *Y. lipolytica* DSM3286 and M7 strains. Subsequently, permeabilization of the yeast cells and improvement of CA production in presence of some detergents like Triton X-100, sodium dodecyl sulfate (SDS), and Tween 80 with different concentrations will be investigated, as well as changes in the morphology and surface structure of yeast cells in the presence and absence of surfactant study by light and scanning electron microscope methods.

Materials and Methods

Microorganisms and Culture Conditions

Y. lipolytica DSM3286 strain was obtained from the culture collection of the DSM, Germany, and *Y. lipolytica* M7 strain was isolated from poultry meat in Isfahan University microbiology lab. Initially, cells were grown in liquid media yeast extract peptone dextrose medium at 29 °C about 24 h and then transmitted in CA production media [18].

The composition of fermentation medium for CA 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, and glucose 100 g per liter. 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 [19]. All of materials were obtained from Merck Company.

Analytical Methods

Determination of CA and glucose was performed using K-CITR enzymatic test kit (Megazyme, Wicklow, Ireland) and glucose kit (Darvash, Tehran, Iran) according to the manufacturer's recommendations. Microbial growth was monitored by optical density and dry weight as previously described [3].

Viability Studies

The viability of yeast cells was determined by the standard plate counting method, and then survival curves for various surfactants like SDS, Tween 80, and Triton X-100 were plotted. The different surfactants concentrations (0.5 to 5%) were added to the CA production media in the mid-log phase.

Microscopic Methods

The yeast cell morphology was investigated by light microscopic method for Triton X-100 treated or untreated samples. Furthermore, scanning electron microscopy (SEM) method was used for yeast cell surface study as described by Galabova et al. [16].

Results

The wild-type strains *Y. lipolytica* DSM3286 and M7 produced 43.3 and 55.5 g/l of CA on the production medium, respectively. The maximum concentration of CA obtained at 144 h after inoculation.

Treatment of the yeast cells with surfactant showed that they could tolerate to 0–2% of Triton X-100, but further concentrations of the surfactant caused lysis of most of the yeast cells. Detergents were added in the mid-log phase (20 h after inoculation) to CA production medium. The results showed that 0.5% Triton X-100 could increase 1.4-fold CA production in comparison to control. However, other detergents like 0.5% SDS decreased CA production of the yeast strains. The 0.5% Tween 80 did not any effect on CA production by *Y. lipolytica* DSM3286, but it had a negative effect on *Y. lipolytica* M7 production (Fig. 1).

Further studies determined that 1–2% concentrations of Triton X-100 could improve alternatively about 50–80% yield of CA production by *Y. lipolytica* M7 and DSM3286. Triton X-100 could not have significant effect on yeast cells growth (Fig. 2 and Table 1).

Light microscopic investigation showed that Triton X-100 could change size and posture of yeast cells, which changed them to small, thin, and tenuous cells in comparison with untreated cells as control (Fig. 3). For deep analysis of morphological changes, SEM method was used. The data showed that untreated cells as control had distinct outlines and smooth surfaces, but treated cells with 1% Triton X-100 had altered cell shapes and folded

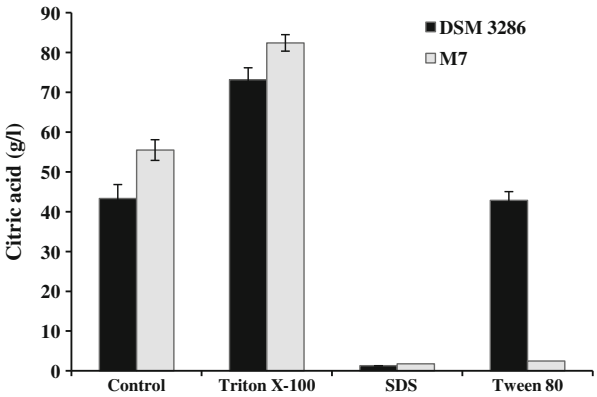

Fig. 1 The effect of various surfactants (0.5% concentration) on citric acid production by *Y. lipolytica* DSM3286 and M7. Control is without surfactant treatment. Error *bars* represent mean of three experiment replicates

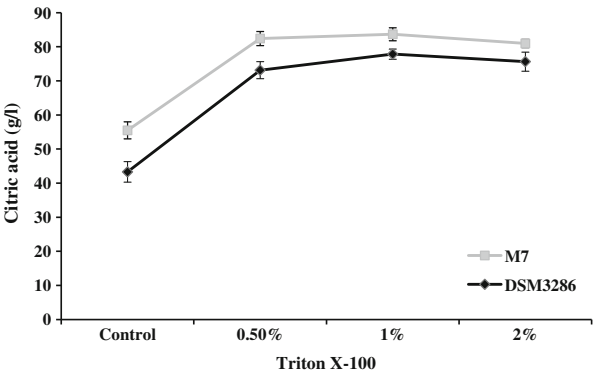

Fig. 2 The effect of different concentrations of Triton X-100 (0–2%) on citric acid production by *Y. lipolytica* DSM3286 and M7. Control is without surfactant treatment. Error *bars* represent mean of three experiment replicates

cell walls. The light microscopic findings were confirmed by SEM micrographs in which the yeast cell structures were smaller and narrower than untreated cells (Fig. 4).

Discussion

The specific concentrations of ionic and nonionic surfactants can be used for destruction of the cytoplasmic membrane of microorganisms. They interfere with cell membrane lipoproteins and solve them, causing release of cytoplasmic agents [14]. The permeabilization processes alters the cell cytoplasmic membrane and leaves the outer membrane intact. Experiments on the permeabilization of microbial cells have been carried out in most studies using a combination of Triton X-100 and another disrupting agent. These processes are employed to determine intracellular enzyme activities as well as protein release from cells [11].

The CA fermentation is an interesting object in the industrial microbiology field, and discovery of new fermentation techniques in order to improve the efficiency of this interesting process is always of importance. Hence, this study focused on improvement of CA production by releasing it from yeast cells using various surfactants, especially Triton X-100.

Papanikolaou et al. [20] observed that CA production of *Y. lipolytica* ACA-DC 50109 is 42.9 g/l with CA yield of 0.56. The wild-type strains *Y. lipolytica* DSM3286 and M7, which were used in this research, produced noticeable amounts of CA (43.3 and 55.5 g/l that their CA yield are 0.43 and 0.55 g/g, respectively) on the production medium. Various detergent agents (Triton X-100, SDS, and Tween 80) in different concentrations (0.5% to 5%) were used as surfactants for permeabilization of *Y. lipolytica* strains and improvement of CA production.

Table 1 Dry weight and citric acid yield (citric acid conversion yields per unit of glucose consumed) in various concentration of Triton X-100

Strain	Parameter	Control	0.5% Triton	1% Triton	2% Triton
Y. lipolytica DSM3286	Dry weight (g/l)	13.08	14.90	12.20	12.38
	Yield (g_{cit}/g_{glc})	0.43	0.73	0.77	0.75
Y. lipolytica M7	Dry weight (g/l)	9.32	11.60	10.40	9.26
	Yield (g_{cit}/g_{glc})	0.55	0.82	0.84	0.79

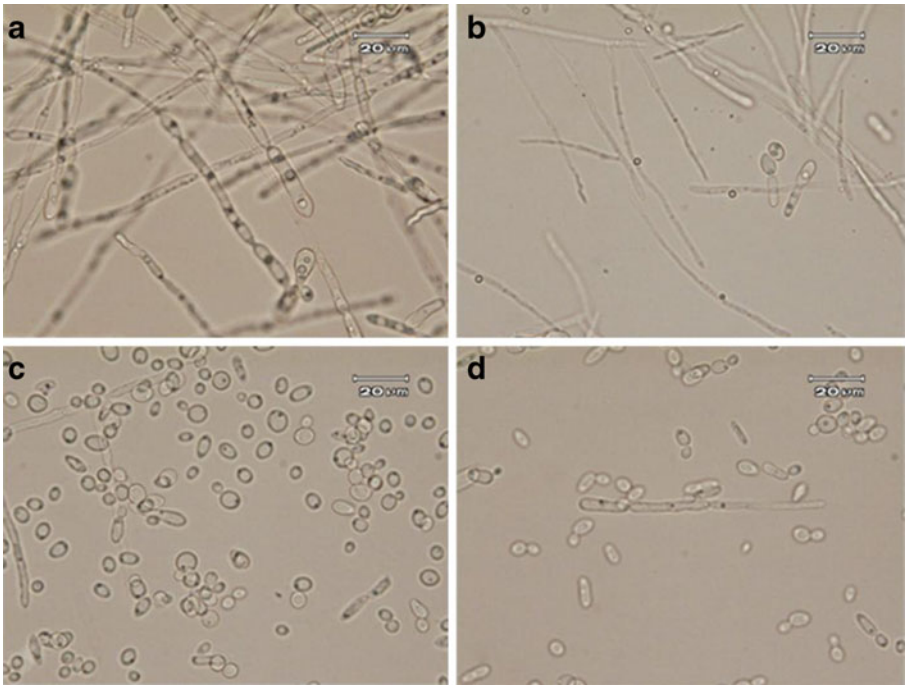

Fig. 3 The light microscopic graphs of yeast cells in absence and presence of 1% Triton X-100 (**a**, **b**: *Y. lipolytica* DSM3286 and **c**, **d**: *Y. lipolytica* M7). Treated cells (**b** and **d**) appear small, thin, and tenuous in comparison with untreated cells as control (**a** and **c**)

Galabova et al. [16] showed that permeabilization of yeast cells in different age with Triton X-100 is maximal in the exponential phase and is effective in the stationary phase. Therefore, the surfactants were added to CA production medium at the mid-log (exponential) phase.

The addition of Triton X-100 in different concentrations (0.5% to 2%) increased 1.4–1.8-fold of the maximum CA quantity achieved for both strains, with final CA concentrations ranging between 75–85 g/l that correspond to CA conversion yields per unit of glucose consumed of ~0.80–0.84 g/g. The 1% Triton X-100 was the best concentration to achieve maximum CA amount. Surprisingly, addition of 1% Triton X-100 after mid-log phase to CA production medium increased CA yield of *Y. lipolytica* DSM3286 and M7 from 0.43 and 0.55 g/g to 0.77 and 0.84 g/g, respectively. When *Y. lipolytica* strains grow on carbohydrate substrates, they have the ability to accumulate high concentrations of citric acid during tricarboxylic acid cycle respiration [4]. Probably, the reason to explain the Triton X-100 effect is that Triton X-100 alters cell permeabilization [14] and, therefore CA accumulated release from the cell to production medium. In addition, Galabova et al. [16] showed intracellular phosphatase production could increase twofold in presence of Triton X-100. Nutan et al. [21] reported increasing of lipase production from 25 to 41 U/ml by treatment with Triton 0.5% X-100. Thus, the permeabilization of yeast cells with Triton X-100 allows the determination of membrane-associated and periplasmic enzymes which are retained in the cells and remain in a naturally immobilized state. The previous findings confirm our results, in which addition of Triton X-100 increased 1.4–1.8-fold of the maximum CA quantity.

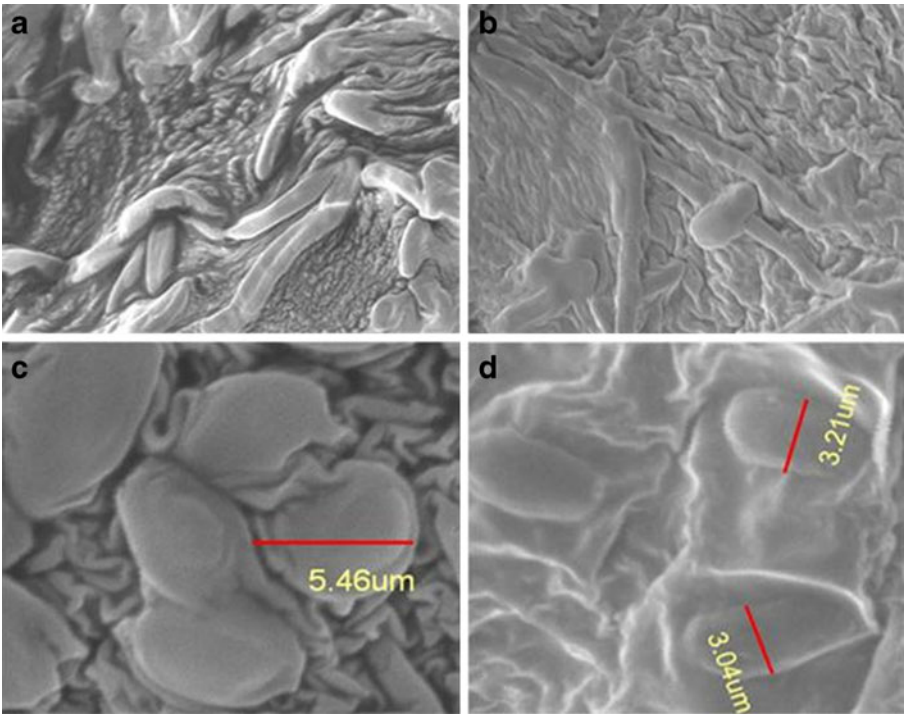

Fig. 4 The scanning electron microscope (SEM) graphs of yeast cell surface in absence and presence of 1% Triton X-100 (**a**, **b**: *Y. lipolytica* DSM3286 and **c**, **d**: *Y. lipolytica* M7). Treated cell structures (**b** and **d**) appear smaller and narrower than untreated cells as controls (**a** and **c**)

Moeller et al. [22] used different fermentation methodologies including batch, fed-batch, repeated batch, and repeated fed-batch cultivations for citric acid production from glucose by yeast *Y. lipolytica*. Their best results were achieved during repeated fed-batch cultivation with CA yields values between 0.51 and 0.65 g/g. In other study, citric acid yield range was 0.50–0.79 g citric acid produced per gram glucose consumed [23]. Furthermore, Rymowicz et al. [4] and Makri et al. [24] results showed that maximum CA yield of *Y. lipolytica* on different glycerol concentrations were 0.62–0.66 g/g. For the first time, surfactant effect on cell permeabilization and CA production relationship were investigated in yeast *Y. lipolytica.* The results proposed that surfactant, especially Triton X-100, can be use to enhance CA production.

In one study, Papanikolaou et al. [25] used longer fermentation time of about 315–330 h and genetically engineered *Y. lipolytica* strains that CA yield values reached to 0.71–0.85 g/g. These strategies are not suitable, because long fermentation time is expensive and genetically engineered strains are not stable in production medium and bioreactor condition. Hence, the results of previous reports and this study show the permeabilization of yeast cells with Triton X-100 is a simple and mild procedure to improve products production in yeasts, especially CA production by *Y. lipolytica*.

The electron microscopic observations show treated yeast cells with low concentration of Triton X-100 does not involve drastic changes in cell integrity, but it can be cause small ultra-structural alterations in the cell wall and membrane. Nevertheless, the growth of cell populations treated by Triton X-100 is slightly depressed in comparison with untreated cells as control. The small ultra-structural alterations in the cell wall and

membrane are probably reason for 1.4–1.8-fold increase in the maximum quantity of CA production, which is confirmed by electron microscopic studies.

In conclusion, the addition of Triton X-100 and same surfactants is a good and cheap strategy to increase CA yields by *Y. lipolytica* in large amounts and industrial production.

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