

INTENSIFICATION OF SURFACTANT SYNTHESIS OF *ACINETOBACTER CALCOACETICUS* IMV B-7241 ON ETHANOL IN PRESENCE OF CITRATE AND FUMARATE

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The influence of the fumarate (C4-dicarboxylic acids precursors of the gluconeogenesis) and citrate (the regulator of lipids synthesizes), optimal pH of biosurfactant synthesis during *Acinetobacter calcoaceticus* IMV B-7241 cultivation on ethanol was studied.

It was determined that the neutralization of the medium by the KOH solution in the process of the IMV B-7241 strain cultivation with the subsequent adding of the fumarate (0.01 %) and citrate (0.01 %) at the end of the exponential growth phase was accompanied by the increasing surfactant synthesis by 1.2-fold comparing to the same indexes without neutralization and by 3.5-fold comparing to the cultivation of bacteria on ethanol without organic acids and pH regulation.

The increasing of the surfactant synthesis in the presence of the fumarate and citrate was caused by the 1.7–7-fold increased activity of the biosynthesis enzymes of glycolipids (phosphoenolpyruvate (PEP)-synthetase and thregalozophosphate-syntase) and aminolipids (NADP+- dependent glutamatedehydrogenaze) as well as the simultaneous functioning of two anaplerotic ways (glyoxylate cycle and phosphoenolpyruvatecarboxylate reaction).

In the previous researches it was shown that the *Acinetobacter calcoaceticus* IMV B-7241 strain, isolated from oil-contaminated soil samples, synthesized the surfactants while growing on hydrophilic and hydrophobic substrates [1]. The conditions of IMV B-7241 strain cultivation on ethanol providing maximum rates of surfactant synthesis were set.

Nowadays, the world's main raw materials for the surfactant synthesis are hydrophobic substrates (usually *n*-hexadecane and liquid paraffin). It should be noted that ethanol is much cheaper and more technological substrate comparing with hydrophobic water insoluble substances. The use of ethanol for the surfactant biosynthesis can reduce the cost of cultivation but the yield of the desired product remains low.

One of the approach to improve the efficiency of microbial technology is to introduce exogenous biosynthesis precursors into the medium – intermediate products of metabolism of the growth substrate (primary metabolites), which are the source for the constructive metabolism or synthesis regulators (inductors) of the target product.

Thus, it was previously shown that introduction of 0.2 % fumarate (precursor of gluconeogenesis) and 0.1 % citrate (regulator of lipid synthesis) at the beginning of the stationary growth phase of *Rhodococcus erythropolis* EK-1 on ethanol was accompanied by the increase of surfactant synthesis by 40–100 % comparing with bacteria growing on medium without fumarate and citrate [2]. The increasing of surfactant synthesis under these conditions was caused to the strengthening of gluconeogenesis that was confirmed by 1.5 and 3.5 fold increase of the isocitratelase and phosphoenolpyruvate (PEP)-synthetase activity (the key enzymes of glyoxylate cycle and gluconeogenesis, respectively), and also lipid synthesis, that could indicate the 1.5-fold reduction of isocitrate dehydrogenase activity [2]. The increase of surfactant concentration by 1.5–1.7-fold in the case of introducing into the nutritive medium of *R. erythropolis* EK-1 with *n*-hexadecane fumarate 0.2% and 0.1% citrate was caused by the intensification of trehalose mycolates synthesis that was confirmed by the 3–5-fold increase of the activity of PEP-synthetase and trehalose phosphate synthase, respectively [3].

As surfactant synthesized by *A. calcoaceticus* IMV B-7241 on ethanol is complex of glyco-, amino- and neutral lipids [1], we assumed that the introduction of fumarate and citrate into the medium, as well as for the strain of *R. erythropolis* EK-1, might be accompanied with the increase of the surfactant biosynthesis.

The aim of the work was to study the possibility of *A. calcoaceticus* IMV B-7241 surfactant synthesis intensification on ethanol in the presence of fumarate and citrate.

The indexes of surfactant synthesis and bacteria growth - concentration of biomass, the surface tension (σ) of cell-free cultural liquid, the conditional surfactant concentration (CSC*, dimensionless), the emulsification index of cultural liquid (E_{24} , %)- were determined as it was described in our previous works.

The amount of the exocellular surfactant (g/L) was determined by the gravimetric method after the extraction from the supernatant of cultural liquid with mixture of methanol and chloroform [1]. The surfactant synthesizing ability was determined as the ratio of the concentration of the synthesized surfactant (g/L) to the concentration of the biomass and expressed in g of surfactant / g of biomass.

At the first stage, similarly to the researchers conducted with *R. erythropolis* EK-1 [2, 3], we studied the influence of precursors (0.1–0.5%) on the surfactant synthesis in the case of their addition into the medium with ethanol in the late exponential and the beginning of the stationary growth phases of *A. calcoaceticus* IMV B-7241. It was determined that the introduction of fumarate and citrate hardly affected the synthesis of surfactants. In this regard, at the next stage of the research the concentration of fumarate and citrate were reduced and organic acids were introduced into the ethanol containing medium at the beginning of *A. calcoaceticus* IMV B-7241 cultivation or in the late exponential growth phase. It was shown that the highest rates of surfactant synthesis were observed in case of simultaneous introduction of fumarate and citrate (0.01 %, Table 1).

Table 1

Surfactant synthesis by *A. calcoaceticus* IMV B-7241 on ethanol in the presence of different concentrations of fumarate and citrate

Concentration of organic acids*	Surfactant concentration, g/L	Emulsification index**, %
Citrate, 0.01	2.6±0.13	91±4
Citrate, 0.02	2.6±0.13	100±5
Citrate, 0.1	1.9±0.10	87±4
Fumarate, 0.01	2.8±0.14	100±5
Fumarate, 0.02	2.5±0.12	87±4
Fumarate, 0.1	2.1±0.10	88±4
Citrate, 0.01 + Fumarate, 0.01	5.0±0.25	89±4
Citrate, 0.02 + Fumarate, 0.02	3.2±0.16	79±4
Citrate, 0.1 + Fumarate, 0.1	2.8±0.14	88±4
Control (without organic acids)	1.7±0.09	88±4

* The introduction of citrate and fumarate was conducted in the late exponential growth phase (48 h).

** Sunflower oil was used as substrate for emulsification.

Thus, the addition of 0.01 % of organic acids in the medium with ethanol in the late exponential growth phase of *A. calcoaceticus* IMV B-7241 was accompanied by the increasing of the concentration of synthesized surfactants almost by 3 times (from 1.7 to 5.0 g/L) comparing with the cultivation of bacteria on the medium without fumarate and citrate (Table 1). In the case of addition of organic acids in concentration 0.02 % the increase of surfactant synthesis to 3.2 g/L was observed (almost two-fold higher than on the medium without organic acids). It should be noted that unlike *R. erythropolis* EK-1 [2, 3], in the process of *A. calcoaceticus* IMV B-7241 cultivation either on ethanol or on ethanol in the presence of fumarate and citrate, the emulsification index of cultural liquid was almost unchanged.

At the next stage the activity of enzymes of surfactant biosynthesis with the introduction of organic acids (0.01 %) into the medium with ethanol was analyzed. The experiments have shown

that under such conditions the 1.7–7.0-fold increase of the activity of all enzymes was obtained excepting isocitratelase, activity of which was almost the same. The increase of PEP-synthetase and PEP-carboxylase activity (more than 7 and 2.4 fold compared with the activity on the medium with ethanol without organic acids) was the most significant. These results may be evidence of the strengthening of glycolipid surfactant synthesis during the strain IMV B-7241 cultivation. The proof of this was more than 3-fold increase of the trehalose phosphate synthase activity – a key enzyme of the biosynthesis of trehalose mycolates. The increasing of isocitrate dehydrogenase and NADP⁺-dependent glutamate dehydrogenase activity and 2-oxoglutarate dehydrogenase absence in the process of growth strain IMB B-7241 on ethanol in the presence of fumarate and citrate may indicate the increasing of aminolipids synthesis.

The patterns of precursors biosynthesis influence on the formation of *A. calcoaceticus* IMV B-7241 surfactant were determined. They differed from those for the strain *R. erythropolis* EK-1 [2]: firstly, the optimum concentration of fumarate and citrate for the strain IMV B-7241 was 10-fold lower; secondly, the presence of organic acids increased only the synthesis of surfactant and thirdly, the effect of simultaneous use of organic acids in the nutritive medium with ethanol pronounced – the concentration of surfactant increased almost by three folds, while for the strain *R. erythropolis* EK-1 it increased only twice.

Our results also differ from those ones in the literature. Firstly, the literature describes the increase in surfactant synthesis in the presence of citrate only, which was introduced into the nutritive medium at the beginning of the cultivation [4, 5]. Secondly, the optimal concentration of citrate was 0.5–1.0 %. With such concentration citrate can be seen not as a regulator of lipid synthesis but as the additional growth substrate. It should be noted that we have not found the information about the intensification of surfactant synthesis by simultaneous introduction into the medium either citrate or C₄-dicarboxylic acids.

The decrease of pH to 5.5–5.7 of the cultural broth was observed on the second day of the *A. calcoaceticus* K-4 cultivation on ethanol and by the end of the cultivation pH decreased to 4.5 – 5.0. The most bacteria transport the salts of organic acids into the cells by the symport with proton. Neutral pH is optimal for such process. Thus, we assumed that the medium neutralization during strain cultivation (and before the addition of organic acids) would be accompanied by the increasing of the surfactant synthesis. The pH of cultural fluid was maintained at a neutral level by addition of 1 N KOH (NaOH) during the process of cultivation.

It was shown that maintaining of the pH at neutral level during bacteria cultivation either on ethanol or ethanol with organic acids was accompanied by the increasing surfactant concentration and surfactant synthesizing ability comparing with the process without pH regulation. It should be noted that the maximum intensification of surfactant synthesis (surfactant concentration 6.0 g/L, surfactant-synthesizing capacity 6.2 g surfactant/g biomass) was observed during the simultaneous introducing of fumarate and citrate into the ethanol medium, as well as the use of KOH solution for the maintaining pH at rate of 7.0. The neutralization of the cultural liquid by sodium hydroxide was accompanied by the decrease of the surfactant synthesis and surfactant-synthesizing capacity by 10–12 % comparing to the indicators obtained with KOH regulation. The cultivating of the IMV B-7241 strain on ethanol in the presence of organic acids and using of KOH as titration agent the emulsification index of the cultural liquid increased (by 7–9 %) comparing with the process without pH regulation.

Further experiments showed that sodium cations were inhibitors of the enzyme activity of the surface-active glyco- (PEP-synthetase) and aminolipids (NADP⁺-dependent glutamate dehydrogenase) biosynthesis by *A. calcoaceticus* IMV B-7241 (Table 2). Thus, the activity of PEP-synthetase and glutamate dehydrogenase decreased by 1.8 and 5 fold respectively in the presence of 50 mM of Na⁺ in the reaction mixture. It was interesting to note that 2-fold reduction of PEP-carboxylase (the enzyme of the anaplerotic reaction filling the pool of C₄-dicarboxylic acids) activity was observed in the presence of sodium cations in the medium with ethanol for strain B-7241 cultivation.

**Sodium cations effect on activity of some enzyme of
A. calcoaceticus IMV V-7241 surfactant biosynthesis**

Concentration of Na ⁺ in the reaction mixture, mM	Enzyme activity* (nmol·min ⁻¹ mg ⁻¹ of protein), % of the control			
	(PEP)-synthetase	PEP- carboxykinase	NADP ⁺ - dependent glutamate dehydrogenase	PEP carboxylase
25	72±3.6	D. d.**	20±1.0	70±3.5
50	55±2.8	95±5.0	20±1.0	60±3.0

*Enzyme activity was determined for the cells being at the beginning of the exponential phase of growth (24 hours). The control (100 %) was enzyme activity without sodium cations in the reaction mixture.

**D. d. was not determined.

These results should be considered in the development of microbial surfactants biotechnology particularly while choosing the titration agent.

Organic acids were introduced into the medium with ethanol as sodium salts while studying the influence of the fumarate and citrate on the surfactant synthesis by strain IMV B-7241. Obviously their replacing with potassium salts may be accompanied by the increase of surfactant synthesis. Clarification of these issues will be studied in our future researches.

Thus, the possibility of the increasing of *A. calcoaceticus* IMB B-7241 surfactant synthesis on ethanol while maintaining the pH at a neutral rate by KOH and addition of fumarate and citrate (0.01 %) in the late exponential phase of growth was established. The obtained results had shown the possibility of the regulation of surfactant biosynthesis by *A. calcoaceticus* IMV B-7241 resulting in increasing of biosurfactant concentration.

References

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