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BIOLOGICALS TECHNOLOGY

Optimization of Repeated-Batch Fermentation of a Recombinant Strain of the Yeast *Yarrowia lipolytica* for Succinic Acid Production at Low pH

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Abstract—The capacity of a recombinant *Yarrowia lipolytica* yeast strain VKPM Y3753 for succinic acid biosynthesis in a laboratory bioreactor at low pH has been studied. The batch and repeated-batch modes of fermentation of the strain were compared. The optimal conditions for repeated-batch fermentation were selected; they resulted in the accumulation of 55.3 g/L of succinic acid and the maximal productivity for this compound, 2.6 g/(L h), while lowering the pH of the broth culture to 3.65 at the end of the biosynthesis process.

Keywords: succinic acid biosynthesis, low pH, repeated-batch fermentation, batch fermentation, *Yarrowia lipolytica*

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INTRODUCTION

Succinic acid (SA), having the form of colorless crystals soluble in water, ethanol, and acetone, was first discovered in 1550 in amber. The compound is characterized by a wide range of industrial applications, in particular, in obtaining intermediates for the production of lacquers and flavorings, as well as food additives and bacteriostatic and neutralizing agents in the food industry. In addition, SA is in demand from manufacturers of protective coatings, surfactants, dyes, detergents, solvents, biodegradable plastics, and substances that stimulate the growth of animals and plants. Due to its saturated linear structure and the presence of two carboxyl groups, SA serves as a source for the production of many chemical compounds, such as 1,4-butandiol, γ-butyrolactone, tetrahydrofuran, adipic acid, n-methylpyrrolidone, and aliphatic esters [1–4].

In connection with the deterioration of environmental conditions, the demand for SA is expected to be increased significantly, since it is possible, for example, to produce a new biodegradable polymer poly-(1,3-propylresorcinol) via its thermal polycondensation with 1,3-propandiol [5]. 1,4-butanediol can also be derived from SA, which is further used, in particular, for the production of biodegradable polybutylmethacrylate [6–8].

Succinic acid can be obtained by different chemical methods, which mainly include paraffin oxidation or the catalytic reduction of maleic acid or maleic anhydride. In the process of catalytic oxidation of paraffins, a mixture of various dicarboxylic acids is produced, from which SA is then recovered and purified by distillation [9, 10]. As early as 1929, the American company National Aniline & Chemical launched the industrial production of SA by electrolytic reduction of maleic anhydride [11]. In SA production by chemical means, nonrenewable raw materials (oil, natural gas) and energy-intensive technologies that require high temperatures and pressures are used. In addition, the problem of waste disposal and rising oil prices necessitate a search for other ways to obtain SA with the use of renewable raw materials [12].

Developments in the field of SA production are currently focused on the use of microorganisms, in particular, bacterial strains that assimilate renewable plant raw material: *Propionibacterium* sp., *Escherichia coli*, *Pectinatus* sp., *Bacteroides* sp., *Ruminococcus flavefaciens*, *Actinobacillus succinogenes*, *Prevotella ruminicola*, *Succinimonas amylolytica*, *Succinivibrio dextrinisolvens*, *Wolinella succinogenes*, *Lactobacillus* sp., and *Cytophaga succinicans* [13–16].

It should be noted that the process of SA production by bacteria occurs in an anaerobic environment with carbon dioxide absorption, and this can be considered an advantage from the point of view of ecology. On the other hand, the use of bacteria is possible only at neutral pH; therefore, it is necessary to use an alkali to neutralize the product, SA. As a result, succinic acid salt is formed and, for SA regeneration, it is necessary

Abbreviations: VKPM—Russian National Collection of Industrial Microorganisms; BC—broth culture; SA—succinic acid.

to use a strong mineral acid, which leads to a cost increase of the final product and the formation of additional waste. One possible way to purify SA is the use of ion-exchange resins. However, while such methods of isolation of a product are easily feasible in small laboratory units, they are not usually applicable on an industrial scale [17].

Intensive work is currently being conducted on the production of yeast SA producers that are able to synthesize it at low pH. For today, the best results are obtained with the yeast *Saccharomyces cerevisiae* SUC-632: SA accumulation—80.1 g/L; SA yield—0.512 g/g glucose at a final pH of 3.0. In this case, fermentation took place with the biomass return (every 48 h) and with the use of chalk as a buffer agent. Such a mode ensured a productivity of 1.67 g/(L h) [18]. The highest result for SA accumulation (160.2 g/L) by the yeast *Yarrowia lipolytica* PGC01003 was obtained by repeated-batch fermentation at pH 6.0 with 5 M NaOH as a titrating agent and glycerol as a carbon source. The SA yield was 0.4 g/g glycerol, and the productivity was 0.4 g/(L h) [19].

The *Yarrowia lipolytica* yeast strain VKPM Y3753 produced in the State Research Institute for Genetics and Selection of Industrial Microorganisms (GosNIIgenetika), when cultured in test tubes on a rich medium containing yeast extract and peptone, is able to accumulate 60.0 g/L of SA for 96 h. The product yield is of 0.57 g/g glucose at a final of pH 3.0 [20].

The goal of this study was to optimize SA biosynthesis by a *Yarrowia lipolytica* strain (VKPM Y3753) in a laboratory bioreactor via the selection of an efficient cultivation mode and its parameters.

EXPERIMENTAL CONDITIONS

The *Yarrowia lipolytica* yeast strain VKPM Y3753 from the National Bioresource Center, Russian National Collection of Industrial Microorganisms (BRC VKPM, GosNIIgenetika), was used throughout the work.

The culture was maintained at 4°C on YPG agar medium with chalk of the following composition, g/L: yeast extract (Springer, France), 10; yeast peptone (Springer), 10; glycerol (Khimmed, Russia), 20; agar (BD, France), 20; Ca_2CO_3 (Khimmed, Russia), 10. Reseeding into fresh medium was performed once a month.

Inoculum for the bioreactors was grown first in test tubes for 48 h at 30°C and 250 rpm in 10 mL of mineral medium of the following composition, g/L: $(NH_4)_2HPO_4$, 7.3; $NH_4H_2PO_4$, 10.89; KCl, 1.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.65; $CaCl_2$, 0.111; citric acid (all products of Khimmed), 1.8; glucose (Roquette, France), 30; L-leucine (DiaM, Russia), 5.0; 4.6 mL of the solution of trace elements (composition of the solution of trace elements, g/L: $CuSO_4 \cdot 5H_2O$, 6.0; KI, 0.088; $MnSO_4 \cdot 5H_2O$, 3.0; H_3BO_3, 0.2; $CoCl_2 \cdot 6H_2O$, 0.955; $ZnSO_4 \cdot 7H_2O$, 42.0; $FeSO_4 \cdot 7H_2O$, 65.0 (all products of Khimmed); concentrated H_2SO_4 (Sigma Tech, Russia), 5.0 mL/L; Biotin (AppliChem, Germany), 0.2 mg/L; thiamine (AppliChem), 1.0 mg/L.

BC from the test tubes was then used as an inoculum for the flasks in the amount of 10% (vol). Cultivation in the flasks was carried out for 24 h under the same conditions in 100 mL of mineral medium of the same composition as in the test tubes.

Bioreactor cultivation was carried out in Marubishi Oh bioreactors (Japan) with an integrated system of management software, MERABIT (Keklab, Russia), on a volume of 1.0 L (working volume of 600 mL). Aeration was carried out by air supply (600 mL/min) at a rotational speed of the stirrer of 700 rpm. The oxygen concentration in the medium was controlled by a Mettler Toledo InPro6800 (Switzerland) sensor. The cultivation was carried out at a temperature of 30°C. The medium was of the same composition as for inoculum growth, but the initial glucose concentration was unique for each mode. The pH value of the medium (the initial value is specified for each experiment in the text) was controlled by the Mettler Toledo InPro3030 (Switzerland) sensor, increasing this value if necessary by adding of 25% NH4OH solution.

Cultivation in batch mode was carried out for 68 h with an initial glucose concentration of 150 g/L and initial pH of 7.28. Starting from the 49th hour, the pH was maintained at 3.6 by the addition of 25% NH_4OH solution. The volume fraction of added inoculum grown in flasks was 30%.

Optimization of the repeated-batch fermentation mode was performed by three different algorithms differing in duration of the "withdrawal-addition" cycle—48, 24, and 8 h. At the periodicities of 48 and 24 h, 70.0% (vol) of BC was withdrawn at the end of the cycle; at the periodicity of 8 h, the withdrawal was 33.3% (vol). After the BC withdrawal, fresh nutrient medium of the same composition and the above indicated volume was added to the bioreactors, but with a glucose concentration that could be fully assimilated by the end of the cycle of cultivation. The initial pH of the medium in the bioreactor at periodicities of 48 and 24 h in each cycle was increased up to 7.0. At the periodicity of 8 h, the initial pH was successively decreased in each new cycle (down to 6.0 in the first and to 4.3 in the last two). The final pH in each cycle decreased to the minimum value at which no inhibition of yeast growth was observed. The procedure of repeated-batch was repeated until a stationary state of the semi-continuous process with optimal values of the initial glucose concentration and pH of BC was established, i.e., until these indicators did not start to reproduce on their own, which happened in the last two cycles of each mode. In the fermentation process, BC samples were taken; the biomass was separated by a MiniSpin Eppendorf (Germany) microcentrifuge at 14100 *g* for

5 min, washed twice with distilled water, and then freeze-dried before weighing. The glucose and SA concentrations were measured in the supernatant.

The glucose content in the supernatant was determined by the spectrophotometric glucose oxidase method with a Diakom Glucose reagent kit (CJSC Diakom—DS, Russia) according to the manufacturer's instruction.

The contents of SA and other organic acids was determined by HPLC with an Alliance (Waters separation module 2695; photodiode array detector 2996, Waters, USA) chromatograph and a YMC-Triart C18 column (4.6 mm × 250 mm, 5 μm, 12 nm, Dr. Maisch, Germany) with a reverse phase. Elution was carried out with an aqueous solution of phosphoric acid (0.1%) at a rate of 1 mL/min. Peaks were recorded at a wavelength of 210 nm.

The specific growth rate of the culture μ (h^{-1}) was calculated with the formula:

$$\mu = \frac{\ln m_2 - \ln m_1}{t_2 - t_1},$$

where m_1 and m_2 are the biomass concentrations, g/L, at times t_1 and t_2, h, respectively.

The specific rate of SA biosynthesis q_p (g SA/g biomass h) was calculated with the formula

$$q_p = \frac{P_2 - P_1}{\frac{m_2 + m_1}{2}(t_2 - t_1)},$$

where P_1 and P_2 are the SA concentrations, g/L, at times t_1 and t_2, h, respectively.

The SA yield per unit of consumed substrate Y (g/g) was calculated with the formula

$$Y = \frac{R}{S},$$

where R is the total amount of acid at the end of cultivation, g, and S is the amount of substrate (glucose) consumed for growth and biosynthesis, g.

The productivity of SA (g acid/(L BC h)) was calculated with the formula

$$Q_p = \frac{P_2 - P_1}{t_2 - t_1}.$$

The dilution rate D (h^{-1}) for repeated-batch fermentations was calculated with the formula

$$D = \frac{V_{rev}}{V_p T},$$

where V_{rev} is the revolving volume of the withdrawn BC/added fresh medium, L; V_p is the working volume of the bioreactor, L; and T is the time of the repeated-batch fermentation, h.

RESULTS AND DISCUSSION

The industrial production of a biotechnological product requires, in addition to a good producer with the desired properties (one that is isolated from natural sources and selected or obtained by genetic engineering methods), information about the many factors that determine the cost of the product. These factors include the cost of raw materials, fermentation duration, and equipment lifetime, as well as the material and energy costs of product extraction. In order to evaluate the possibility of cost reduction, it is necessary to simulate various modes and conditions of production in laboratory bioreactors, as well as to find the optimal parameters of fermentation: feed rate of the culture medium; aeration intensity (in the case of an aerobic process); rotation speed of the stirring device; and the pH level, maximum product concentration, and main carbon source at which there is no inhibition of the culture.

An accurate evaluation of the efficiency of a biotechnological production can be carried out only based on the determination of the main criteria (parameters) by which different fermentation processes are to be compared. It is presently customary to use such criteria as the concentration of the product in BC (g/L); the product yield (economic coefficient), which is defined as the ratio of the amount of product produced to the amount of consumed substrate (g/g or %); and the process productivity, which is characterized by the amount of product obtained per unit of working volume of the bioreactor per unit of time (g/(L h)). By comparing these criteria of effectiveness, it is possible to choose the modes of yeast cultivation in a laboratory bioreactor that are the closest to optimal from an economic point of view. The results can be used to scale the production process from a model bioreactor to a pilot plant.

Fermentation in Batch Mode

A simple batch culture containing an initial limited amount of nutrient substrate is a closed system, in which the rate of biomass growth tends to zero, due either to lack of substrate or intolerance to the product during its accumulation. Such systems are always in an unstable state [21]. With an examination of the causes of the unstable state, it is possible to determine the direction of optimization of the cultivation mode of strains producing, in particular, SA.

Figure 1 shows the dynamics of biomass concentration, glucose consumption, and SA accumulation, as well as the dissolved oxygen and pH of the medium upon batch cultivation of the *Yarrowia lipolytica* strain VKPM Y3753 in a bioreactor. For the first 45 h, the culture was in exponential phase, while maintaining the maximum specific growth rate (μ_{max}) 0.076 h^{-1}. Then came the stationary phase, which was reflected in the respiration rate of the culture (see Fig. 1b); at

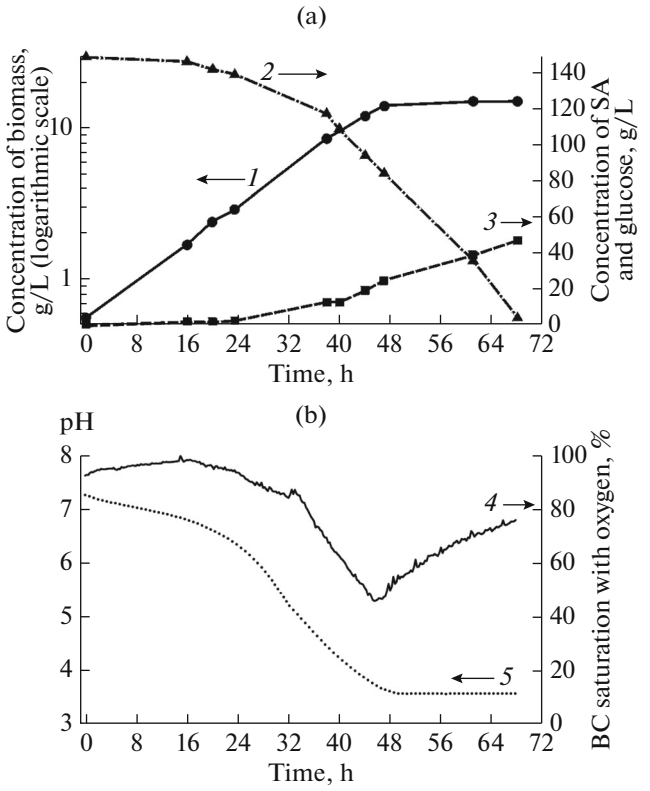

Fig. 1. Changes in the parameters of SA synthesis by the *Y. lipolytica* strain VKPM Y3753 in batch cultivation of the producer in a bioreactor. (a) Dynamics of biomass accumulation (*1*), glucose content (*2*), and SA content (*3*); (b) dynamics of oxygen concentration (*4*) and pH (*5*).

the moment of slowed growth, the glucose concentration was 94 g/L, the SA concentration was about 25 g/L, and the pH dropped to 3.8. It is known that this strain, when cultured in test tubes on a nutrient-rich YPD medium, is able to accumulate 60 g/L of SA in 96 h [20]. On this basis it can be assumed that SA does not inhibit producer growth at a concentration of 25 g/L and that the slowdown of biomass accumulation in the bioreactor is probably due to the low pH of the medium.

SA biosynthesis occurred during the entire process of cultivation in the bioreactor; the maximum specific rate of biosynthesis $q_{p\max}$ was observed from 40 to 47 h and was 0.15 g/(g h) (Fig. 2). By the end of the cultivation (68 h), the SA accumulation reached 47.1 g/L (see Fig. 1a). The average productivity for the entire process was equal to 0.69 g/(L h); the product yield was 0.32 g/g.

On the basis of these results, it can be concluded that, for intensification of SA biosynthesis, it is necessary for the culture to remain as long as possible in the exponential phase of growth at a high biomass concentration, and there is no need to sustain the culture at a pH below 3.8 for a long time.

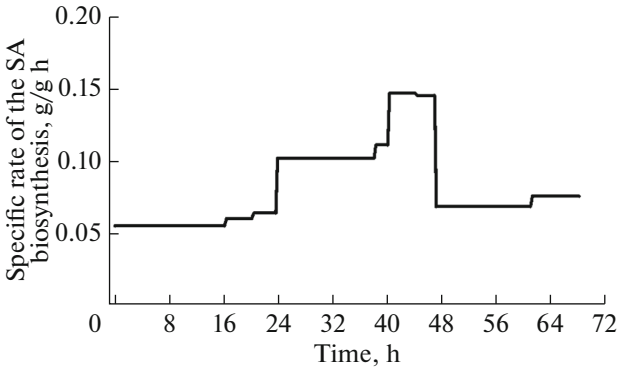

Fig. 2. Change of specific rate of SA biosynthesis by the *Y. lipolytica* strain VKPM Y3753 (average values in the intervals between adjacent samplings).

Fermentation in Repeated-Batch Mode

Based on the results of cultivation in batch mode, it was decided to conduct a study of fermentation in the repeated-batch mode. The latter not only allows continuous provision of the culture with substrate but also dilution of the medium, an increased pH, and an opportunity for the culture to return to the stage of maximum growth. This method of cultivation makes it possible to conduct the process in a virtually continuous mode with periodic removal metabolic products of microorganisms from the bioreactor, which may have an inhibitory effect, thereby resulting in higher productivity of this process compared to other modes.

The main objective of this part of the study was to determine the optimal algorithm of cultivation (the volume of the withdrawn BC and frequency of the withdrawal-addition). It was also necessary to choose a glucose concentration in the medium that is fully consumed by the end of the period, because the presence of glucose can negatively affect the process of SA isolation and purification. It was also important to choose an initial pH that would allow the culture the maximum growth rate and to set the lowest possible pH in the withdrawn BC.

With culturing at periodicities of 48 and 24 h, the dilution rate (D) was 0.015 and 0.03 h^{-1}, respectively. With batch mode of fermentation, it was found that the maximum specific growth rate of the culture (μ_{max}) was equal to 0.076 h^{-1}, which was significantly higher than the speed of dilution characteristic for the repeated-batch modes. Therefore, the biomass concentration at the end of each period increased as long as the system was not in a stationary state similar to such a stationary state in chemostate, which is characterized by constant concentrations of biomass, SA and glucose at the beginning and at the end of each period (Table 1).

When comparing the results of these two repeated-batch modes, it turned out that the average productivity was 0.95 g/(L h) at the 24 h periodicity

Table 1. Biomass concentration with repeated-batch mode of cultivation

Duration of the cycle, h	Duration of fermentation, h	The number of cycles	Concentration of the biomass, g/L			
			beginning of the first cycle	end of the first cycle	beginning of the last two cycles	end of the last two cycles
48	144	3	2.5	8.1	4.5	17.0
24	144	6	2.5	9.8	5.5	23.0
8	48	6	8.5	17.0	18	27.5

Table 2. Criteria of the efficiency of various fermentation modes

The criterion of effectiveness	Batch mode	Duration of the cycle at the repeated-batch mode		
		48 h	24 h	8 h
Withdrawn volume, %	100.0	70.0	70.0	33.3
The duration of fermentation (the time of the withdrawal of 100% working volume of bioreactor), h	68.0	68.6	34.3	24.0
Dilution rate, h^{-1}	–	0.015	0.030	0.042
pH at the end of fermentation	3.60	3.89	3.98	3.65
Average efficiency for SA, g/(L h)	0.69	0.57	0.95	2.60
SA yield for consumed glucose, g/g	0.32	0.26	0.26	0.34
Final concentration of SA, g/L	47.1	35.3	31.3	55.3

and 0.57 g/(L h) at the 48 h periodicity. The SA yield in both cases was the same, 0.26 g/g, which indicates the advantages of reducing the period of repeated-batch mode. The final SA concentration at the period of 24 h was 31.3 g/L, which was below 35.3 g/L, the concentration at the 48 h periodicity. This is due to the more frequent dilution of BC with the same volume of fresh medium (420 mL).

Given the results, it was decided to further reduce the duration of the withdrawal–addition but, at the same time, to reduce the effect of the dilution of the fresh medium on the final concentration of the product by reducing the volume of withdrawn BC and the added fresh medium. Further repeated-batch cultivation was performed with a periodicity of 8 h, renewing 33.3% of the volume of culture fluid; the rate of dilution was 0.042 h^{-1}, which is less than the maximum specific growth rate (as with the 24 and 48 h periodicities).

Figure 3 shows how biomass and SA gradually accumulated in BC during each period until the establishment of a stationary state.

With this cultivation mode, the culture for the first 6 h was in the exponential phase, and the decline phase of growth began in the last 2 h before the medium renewal. This time period was characterized by the most active SA biosynthesis.

The average SA productivity in this mode was 2.6 g/(L h), which is almost four times higher than the average productivity in the batch mode of cultivation and confirms the advantage of the reduction of periodicity of the repeated-batch fermentation. The SA yield amounted to 0.34 g/g; the final concentration in the medium was 55.3 g/L. It cannot be said that the proposed mode provides a significant increase in the SA yield or its final concentration in comparison with the batch mode, but the former has a clear advantage in productivity and, consequently, in the duration of fermentation (Table 2).

Thus, as a result of this work, it was possible to show the advantage of repeated-batch mode of cultivation in comparison with batch mode, as well as the possibility of increasing the efficiency of biosynthesis

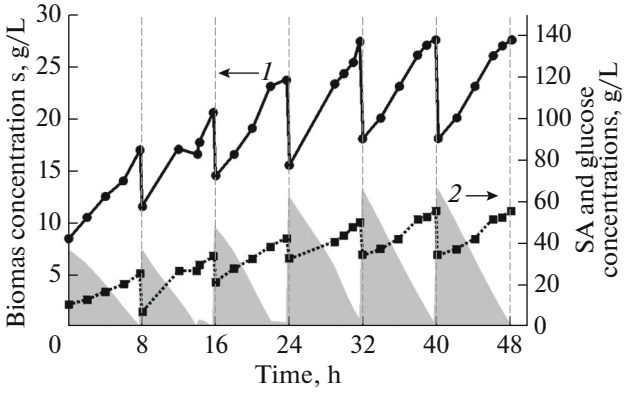

Fig. 3. SA synthesis by the *Y. lipolytica* strain Y3753 in repeated-batch mode with a periodicity of 8 h. (*1*) Biomass concentration; (*2*) SA concentration; gray—glucose concentration.

through the selection of the algorithm for mode implementation. The optimized cultivation process can be used for industrial SA production on the basis of the *Y. lipolytica* yeast strain Y3753.

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