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EXPERIMENTAL ARTICLES

# Characteristics of the Growth on Rapeseed Oil and Synthesis of Citric and Isocitric Acids by *Yarrowia lipolytica* Yeasts

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**Abstract**—The native strain *Yarrowia lipolytica* VKM Y-2373 grown in a complete medium exhibited the maximum lipase activity at the concentration of rapeseed oil of at least 5.0 g/l. In the course of yeast growth, no considerable changes were observed in the glycerol concentration, the proportions of the major free fatty acids formed via oil hydrolysis, or the fatty acid composition of oil. Under nitrogen limitation of cell growth, the accumulation of citric acids reached 77.1 g/l with predominance of isocitric acid at pH 6.0, whereas at pH 4.5, almost equal amounts of citric and isocitric acids were produced. Cultivation of the mutant strain *Y. lipolytica* N 1 at pH 4.5 resulted in the predominant accumulation of citric acid (66.6 g/l) with an insignificant amount of isocitric acid. In the period of intense acid synthesis, high production of lipase was observed.

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*Key words: Yarrowia lipolytica*, citric acid, isocitric acid, plant oils.

In recent years, plant and animal fats have attracted increased interest as renewable bioresources which can be obtained in any country.

Plant oils, rapeseed oil in particular, are excellent substrates for biotechnological processes. However, the scarce data on the growth of yeasts and other microorganisms on plant oils and the synthesis of valuable products are mainly concerned with the production of microbial lipids [1–3] and lipases [2, 4].

The first stage in the utilization of plant oils involves their hydrolysis by extracellular lipases, which results in the formation of glycerol and fatty acids. The recent finding that lipases are also able to perform the reverse reaction of acyl glycerol synthesis, makes it possible to use lipases for the production of stereospecific glycerols with given properties [2]. In spite of the increasing interest in commercial lipase production, the mechanisms of induction and regulation of lipase activity remain unclear. Intensive studies on microbial lipases were carried out at the Institute of Microbiology, Russian Academy of Sciences (E.L. Ruban and I.S. Zvyagintseva) and at the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (laboratory headed by I.S. Kulaev).

Yeasts *Yarrowia* (*Candida*) *lipolytica* are suitable objects for the study of growth characteristics and the formation of valuable products from plant oils, since (1) they exhibit high activity of exolipase due to their taxonomic position [5]; and (2) they are capable of the biosynthesis of organic acids from hydrophobic aliphatic compounds such as *n*-alkanes [6] and fatty acids [7].

We have previously shown that all the strains of yeasts *Y. lipolytica* deposited with the collection of the Laboratory of Aerobic Metabolism of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences grow well in the media containing rapeseed oil as the sole carbon source [8]; however, the strains differ in their exolipase activities. Under nitrogen limitation of cell growth, most of the strains are able to excrete considerable amounts of citric acids. Strains that are characterized by increased lipase activity and by the capability for high production of citric (CA) and isocitric (ICA) acids were chosen for further studies.

The goal of the present work was to study the growth parameters and the synthesis of CA and ICA by both the native strain *Y. lipolytica* VKM Y-2373 and its mutant *Y. lipolytica* N 1 cultivated in media with rapeseed oil.

## MATERIALS AND METHODS

The study was carried out with the native strain *Yarrowia lipolytica* VKM Y-2373 (704) and its mutant N 1 (187/1). These strains were known to exhibit high

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lipase activity and the capability for CA and ICA overproduction. The strains were maintained at 4°C on Reader agar supplemented with 1% paraffin; transfers of stock cultures were performed every three months. Mutant N 1 was obtained by the treatment of the native strain with nitrosomethyl urea and selected as a producer of CA from *n*-alkanes [9].

Rapeseed oil contained the following fatty acids (% of the total fatty acids): $C_{16:0}$, 4.0; $C_{18:0}$, 1.2; $C_{18:1}$, 58.8; $C_{18:2}$, 28.1; $C_{18:3}$, 5.9.

The yeasts were cultivated in an ANKUM-2M fermentor (3 l) with an initial working volume of 1.5 l or in a 10-l fermentor with an initial working volume of 6.0 l. Temperature (28.0 ± 0.1°C) and $pO_2$ (55–60% of air saturation) were maintained automatically; pH was adjusted with 20% NaOH to the values indicated in the text. The medium had the following composition (g/l): $(NH_4)_2SO_4$, 3.0; $KH_2PO_4$, 2.0; $K_2HPO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 1.4; $Ca(NO_3)_2$, 0.4; NaCl, 0.5; trace elements according to Burkholder [10]; thiamine, 0.5 mg/l; and rapeseed oil concentration, as indicated in the text. Cultivation was performed for 96 h.

The procedures used for biomass determination, analyses of oil content, and fatty acid composition of the extracellular lipids, and assays of nitrogen and glycerol were described earlier [8]. CA and ICA were analyzed by high-performance liquid chromatography (HPLC) with an HPLC chromatograph (LBK, Sweden) on an Inertsil ODS-3 reversed-phase column (250 × 4 mm) (Elsiko, Russia) at 210 nm; 20 mM phosphoric acid was used as a mobile phase with the flow rate of 1.0 ml/min; the column temperature was maintained at 35°C. Organic acids were identified by using the standard CA solution (Boehringer Manheim, Germany).

The calculations of specific growth rate (μ), specific rate of the synthesis of citric acids (ICA + CA) ($q_p$), mass yield of acids ($Y_p$), and energy yield of acids ($\eta_p$) were described earlier [8]. These parameters were calculated from the total amount of acids produced taking into account the dilution factor.

Quantitative analysis of lipase activity was carried out using the titrimetric method developed earlier [8]. One unit of lipase activity (U) was defined as the amount of enzyme required for the formation of 1 μmol of fatty acids per 1 min at 37°C. Specific lipase activity was expressed in units per 1 ml of culture liquid (U/ml).

## RESULTS AND DISCUSSION

**Growth of the native strain *Y. lipolytica* VKM Y-2373 and lipase production in complete medium with rapeseed oil.** The native strain *Y. lipolytica* VKM Y-2373 was cultivated in the complete medium containing 20 g/l of rapeseed oil and 3.0 g/l of $(NH_4)_2SO_4$ in a 3-l fermentor; pH (5.0 ± 0.1) was adjusted automatically. The typical time course of *Y. lipolytica* growth is shown in Fig. 1. Lipase activity rapidly increased during the first hours of cultivation; the synthesis of lipase continued concurrently with the yeast growth for 10 h as rapeseed oil was consumed (Fig. 1a). Around the 15th hour of cultivation, when the concentration of rapeseed oil in the medium decreased below 5.0 g/l, lipase activity dropped sharply; in this case, the residual nitrogen concentration remained at a high level (data not shown). It should be noted that lipase activity was restored after the introduction of extra oil into the medium (3.8 g/l); it then increased and reached 47.0 U/ml by the 18th hour of cultivation. This value is comparable with those obtained for other native strains of *Y. lipolytica* [11]; however, it is considerably lower than those revealed for genetically modified mutant strains of *Y. lipolytica* [2, 4]. Therefore, to obtain high lipase activity of *Y. lipolytica*, the concentration of rape-

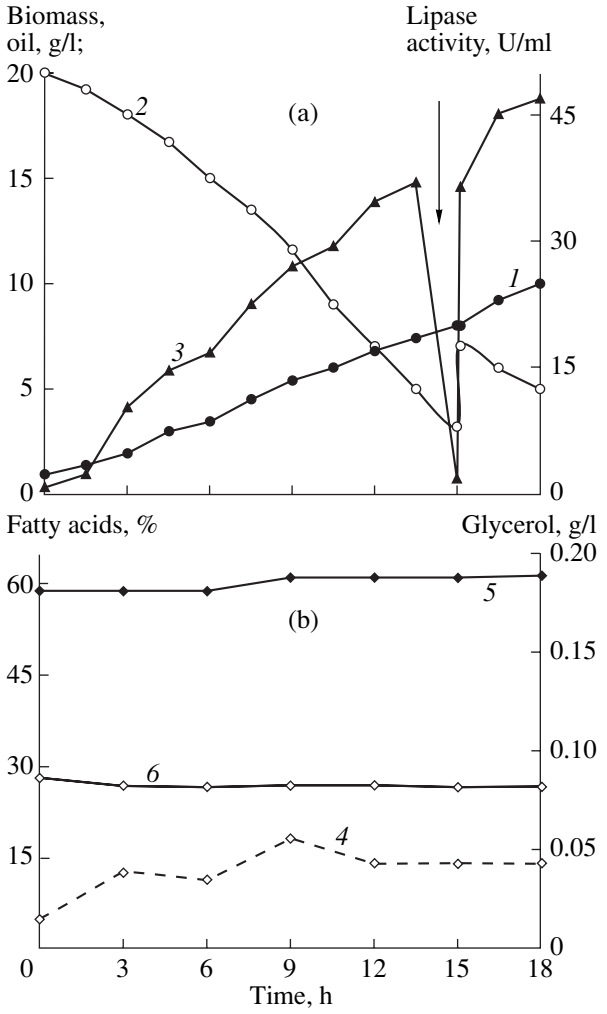


**Fig. 1.** The time courses of (a) cell growth, oil consumption, and lipase synthesis and (b) accumulation of glycerol and fatty acids during cultivation of the native strain *Y. lipolytica* VKM Y-2373 in complete medium at pH 5.0. Curves: (*1*) biomass; (*2*) oil; (*3*) lipase; (*4*) glycerol; (*5*) $C_{18:1}$; (*6*) $C_{18:2}$. The arrow indicates the time of oil addition.

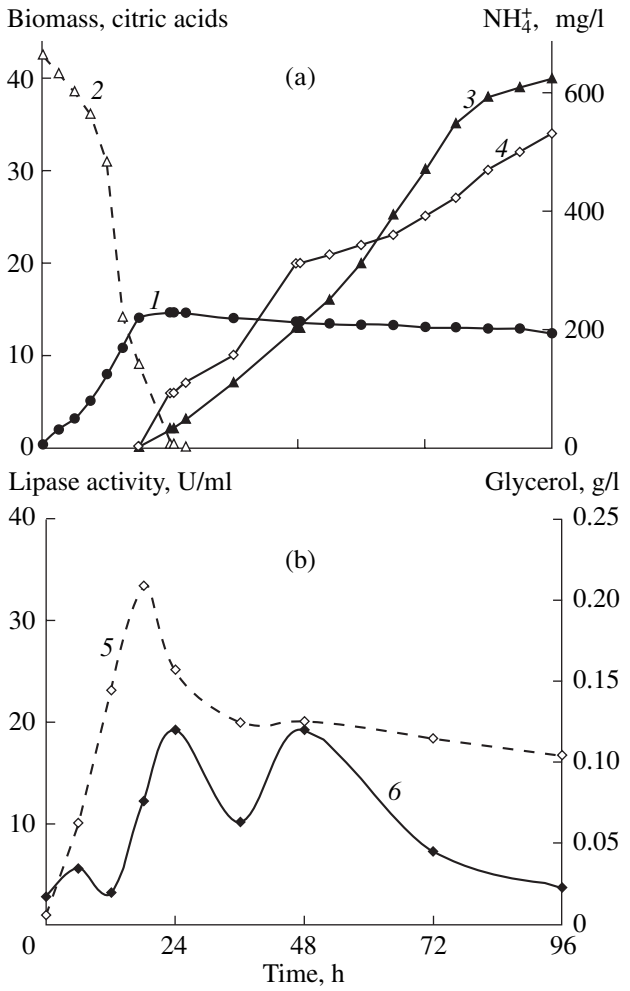


**Fig. 2.** The time courses of (a) cell growth and synthesis of citric acids and (b) lipase production and glycerol accumulation during cultivation of the native strain *Y. lipolytica* at pH 4.5. Curves: (*1*) biomass; (*2*) nitrogen; (*3*) CA; (*4*) ICA; (*5*) lipase; (*6*) glycerol.

seed oil in the medium should be above 5.0 g/l; in further experiments, the residual concentration of rapeseed oil was maintained at this level.

The maximal specific growth rate ($\mu_{max}$) calculated from the linear segment of the growth curve reached 0.281 $h^{-1}$, a value comparable with the literature data obtained for *Y. lipolytica* grown on fatty acids [1–3]. Mass cell yield from oil consumed ($Y_{x/s}$) comprised 0.638, which was lower than the values obtained for various strains of *Y. lipolytica* grown on fatty acids ($0.9 \pm 0.2$ g/g) [1–3]; this can be explained by the low nitrogen concentration in our experiment, which was insufficient for active functioning of protein-synthesizing systems.

In the course of yeast cultivation, no marked changes were revealed either in glycerol concentration (0.035–0.056 g/l) (Fig. 1b) or in the composition of free fatty acids (data not shown). Since no accumulation of any products of oil hydrolysis was observed, it can be assumed that the consumption of glycerol and fatty acids by yeasts *Y. lipolytica* occurred simultaneously. The level of predominant fatty acids, oleic ($C_{18:1}$) and linoleic ($C_{18:2}$), remained relatively constant in the course of yeast growth (58.8–61.3 and 26.6–28.1%, respectively) (Fig. 1b). A similar pattern was observed for strain *Candida lipolytica* 1094 grown on corn oil [1]. Contradictory data were obtained with strain *Y. lipolytica* LGAM S(7) grown on a mixture of unsaturated and saturated fatty acids: this strain consumed mainly oligo- and polyunsaturated fatty acids, which resulted in considerable changes in the fatty acid composition of oil in the course of cultivation [3]. In this case, saturated fatty acids prevailed in intracellular lipids of yeasts in spite of the predominant consumption of unsaturated fatty acids.

**Growth of the native strain *Y. lipolytica* VKM Y-2373 and synthesis of citric acids under nitrogen limitation.** To study the production of citric acids by *Y. lipolytica* VKM Y-2373 under nitrogen limitation, yeasts were grown in a 10-l fermentor at pH 4.5 or 6.0. The initial concentration of rapeseed oil in the medium was 30 g/l; pulsed additions of oil were made when oil concentration decreased below 5.0 g/l. The parameters of the cell growth and acid synthesis at pH 4.5 and 6.0 are shown in Figs. 2 and 3, respectively.

At pH 4.5, active cell growth during the first 24 h was followed by the transition to the stationary phase caused by nitrogen exhaustion (Fig. 2). This event coincided with the beginning of acid synthesis. By the end of cultivation (96 h), concentrations of ICA and CA reached 34 and 40 g/l, respectively; the ICA : CA ratio was 1 : 1.18. Yield of the acids from oil consumed ($Y_{CA + ICA}$) was 1.42. During exponential growth of *Y. lipolytica*, a considerable amount of lipase was produced (33.3 U/ml), which was comparable with the values obtained for this strain grown at pH 5.0. Lipase activity decreased somewhat under nitrogen limitation of cell growth; however, it remained at a rather high level (16.7–21.6 U/ml) throughout the period of acid synthesis. It can be assumed that the content of proteins, in particular of lipase, in the culture broth decreased under nitrogen deficiency. The glycerol concentration remained below 0.120 g/l. No marked changes in the composition of fatty acids was observed in the course of yeast growth (data not shown); oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids prevailed in all samples.

A change in the pH value from 4.5 to 6.0 showed no marked effect on the biomass accumulation and acid production (Fig. 3). By the end of cultivation (96 h), the total concentration of acids and the acid yield from oil consumed ($Y_{CA + ICA}$) amounted to 77.1 g/l and 1.32 g/g, respectively. However, the composition of citric acids depended considerably on the pH value; at pH 6.0, the concentrations of ICA and CA were 55.4 and 21.7 g/l,

respectively, with a ICA : CA ratio of 2.55 : 1, whereas at pH 4.5, the ratio of ICA to CA was 1 : 1.18. Similar patterns were observed earlier with this strain grown on ethanol; at pH 4.5, yeasts actively produced both CA and ICA (the ICA : CA ratio was 1 : 1.7), whereas at pH 6.0, ICA synthesis prevailed (the ICA : CA ratio was 2 : 1) [12, 13]. It is known that CA transport across the membrane is stimulated by low pH values [14], whereas ICA transport is independent of pH; this may explain why at pH 4.5 the ratio of ICA to CA changed in favor of CA.

A change in pH from 4.5 to 6.0 showed no marked effect on the lipase synthesis and glycerol accumulation. According to literature data, *Y. lipolytica* exhibited high lipase activity within a wide range of pH (3.0–6.0) [4, 8, 11].

The parameters of ICA overproduction were calculated for *Y. lipolytica* grown at pH 6.0; the specific rate of ICA synthesis ($q_{ICA}$) amounted to 0.049 g ICAg cells$^{-1}$ h$^{-1}$, which was in agreement with the value calculated for this strain grown on ethanol as the source of carbon and energy [12, 13]. The volume productivity of the ICA production was as high as 0.75 g ICA l$^{-1}$ h$^{-1}$, which was comparable with the data presented in the literature [12, 15]. The yield of ICA from oil consumed ($Y_{ICA}$) was 0.95; earlier, an ICA yield of 0.60 had been obtained for this strain grown on ethanol [12, 13]. According to Peltsmane et al. [15], mutant strains of *Y. lipolytica* grown on *n*-alkanes produced ICA with a higher yield (1.2). It should be noted that it is incorrect to compare the mass yields of ICA ($Y_{ICA}$) obtained on different substrates, since equal quantities of various organic substrates possess different energy capacities. That is why we calculated the energy yield of ICA synthesis ($n_{ICA}$), which was equal to 0.26 in the case of *Y. lipolytica* VKM Y-2373 grown on rapeseed oil. The energy yields of ICA synthesis calculated from literature data for yeasts grown on ethanol and *n*-alkanes amounted to 0.22 [12, 13] and 0.26 [15], respectively.

**Growth and citric acid synthesis by the mutant strain *Y. lipolytica* N 1 under nitrogen limitation at pH 4.5.** As can be seen from Fig. 4, accumulation of citric acids started after the exhaustion of nitrogen in the medium. By the end of cultivation (96 h), the total concentration of citric acids reached 72.8 g/l with the mass yield ($Y_{CA+ICA}$) of 1.85. It should be noted that the ICA content was insignificant (6.2 g/l); the ratio of CA to ICA was 11.7 : 1. The parameters of the CA overproduction by the mutant *Y. lipolytica* N 1 grown at pH 4.5 were as follows: the productivity of the process (C) reached 0.862 g CA l$^{-1}$ h$^{-1}$; the specific rate of CA synthesis ($q_{CA}$) amounted to 0.086 g CA g cells$^{-1}$ h$^{-1}$; the mass yield of CA from rapeseed oil ($Y_{CA}$) was as high as 1.68.

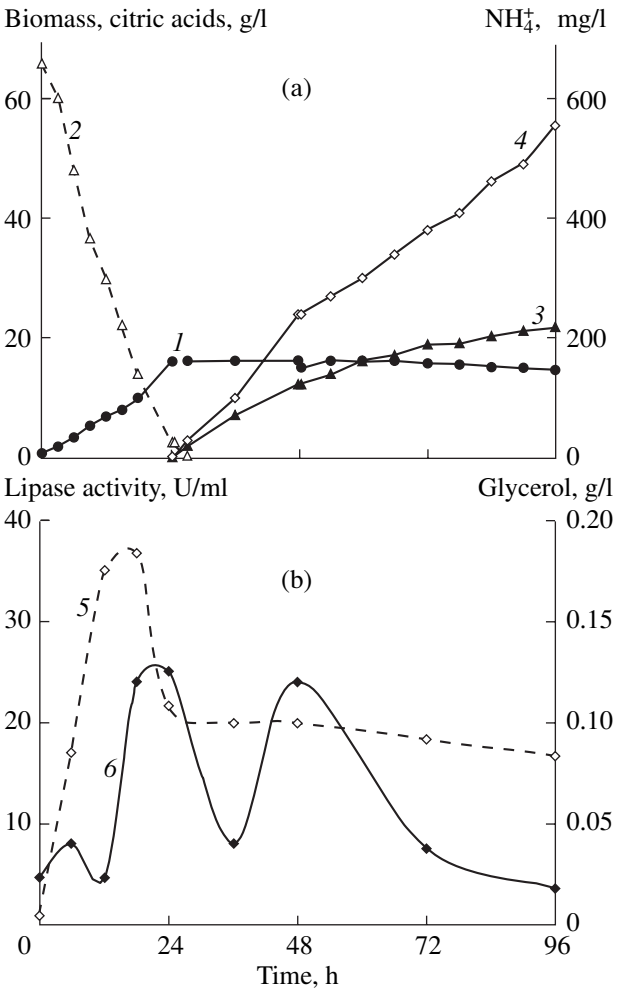


**Fig. 3.** The time courses of (a) cell growth and synthesis of citric acids and (b) lipase production and glycerol accumulation during cultivation of the native strain *Y. lipolytica* at pH 6.0. Curves: (*1*) biomass; (*2*) nitrogen; (*3*) CA; (*4*) ICA; (*5*) lipase; (*6*) glycerol.

The mutant strain *Y. lipolytica* N 1 produced high amounts of lipase; the lipase activity reached 40 U/ml in the exponential growth phase and comprised 22–24 U/ml during the period of acid synthesis. The glycerol content was below 0.125 g/l (Fig. 4).

Thus, the results obtained indicate that rapeseed oil is a promising carbon source for the *Y. lipolytica* growth, lipase production, and the synthesis of citric acids. The native strain *Y. lipolytica* Y-2373 produced the maximum amount of lipase (47.0 U/ml) during unlimited growth at pH 5.0. When grown under nitrogen limitation at pH 6.0, strain *Y. lipolytica* Y-2373 produced citric acids up to 77.1 g/l with the predominance of ICA (55.4 g/l), whereas at pH 4.5, the production of ICA and CA comprised 34 and 40 g/l, respectively. The mutant *Y. lipolytica* N 1 grown at pH 4.5 produced citric acids up to 72.8 g/l with predominance of CA (66.6 g/l). Yeasts *Y. lipolytica* were characterized by a high pro-

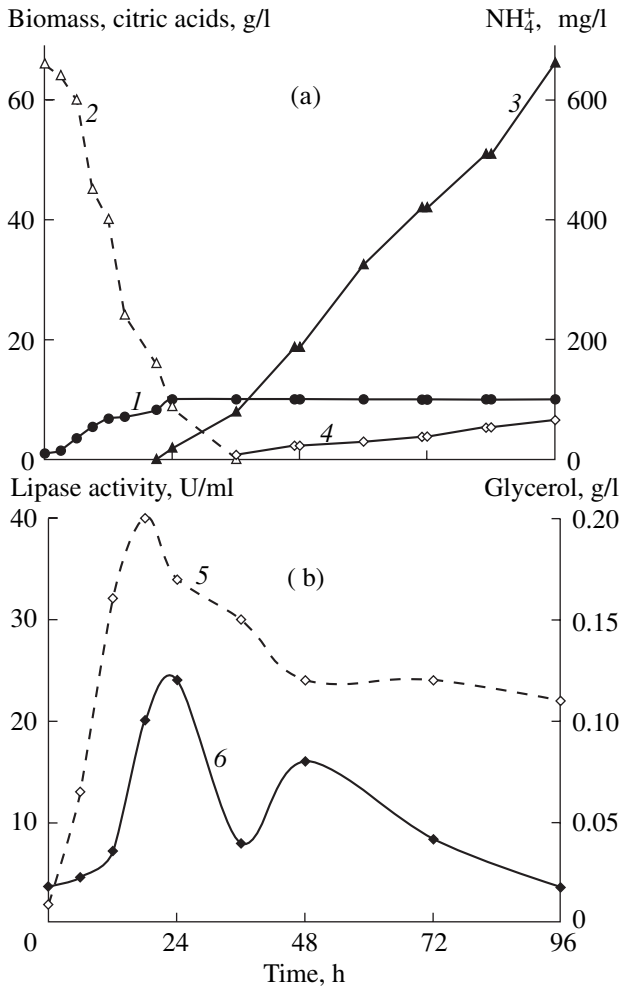


**Fig. 4.** The time courses of (a) cell growth and synthesis of citric acids and (b) lipase production and glycerol accumulation during cultivation of the mutant strain *Y. lipolytica* N 1 at pH 4.5. Curves: (*1*) biomass; (*2*) nitrogen; (*3*) CA; (*4*) ICA; (*5*) lipase; (*6*) glycerol.

duction of lipase (22–24 U/ml) in the period of acid synthesis on condition that oil concentration was above 5.0 g/l.

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