Isocitric Acid Production from *n*-Alkanes by *Candida catenulata*Tadaatsu NAKAHARA, Atanas C. KAIMAKTCHIEV,* Masako OOGAKI-CHINO,**
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Received February 4, 1987

Candida catenulata CBS 1904, the previous type strain of *C. ravauii*, was selected as the best strain for the production of *threo*-D_s-isocitric acid from water-insoluble carbon sources, non-carbohydrates. The addition of surfactants, lipophilic polyoxyethylene nonyl phenyl ethers, was essential for the acid production, because the cell surface of the strain was less lipophilic. *n*-Alkanes, ranging from C₁₁ to C₁₄, gave the acid in high yields. The acid was produced in a jar fermentor in an about 90% yield on a weight basis as to *n*-tetradecane supplied. The acid was easily recovered, as crystals of its monopotassium salt, from the concentrated culture broth filtrate.

We previously described the production of *threo*-D_s-isocitric acid by *Candida ravauii* Langeron et Guerra from glucose, and showed that all the tested strains of the species were able to excrete isocitric acid but little citric acid, and a mutant, selected as the best strain, accumulated the acid in an about 50% yield on a weight basis as to glucose supplied.¹⁾ *C. ravauii* in the second edition of The Yeasts²⁾ has recently been reported to be conspecific with and incorporated into *C. catenulata* Diddens et Lodder in the third edition.³⁾ We tried to achieve the production of the acid from water-insoluble carbon sources, non-carbohydrates, instead of glucose, because recovery of the acid from the culture broth was thought to be easy to the absence of water-soluble impurities originating from raw materials. This paper describes experiments on the acid productivities of the different strains of *C. catenulata* from several-insoluble carbon sources and on the culture conditions to increase the acid yield. *C. catenulata* seemed to consist of a strain group of producers of isocitric acid and another group of non-

producers of the acid, and the former group corresponded to the previous *C. ravauii*.²⁾ Since the cell-surfaces of the tested strains were found to be less lipophilic, this fermentation was markedly improved by the addition of surfactants suitable for acid production and by the use of a jar fermentor with agitation.

MATERIALS AND METHODS

Microorganisms. Five strains of *C. catenulata*, CBS 565, 1904, 2015, 2819 and 6174, were kindly supplied by the Centraalbureau voor Schimmelcultures, the Netherlands. *C. catenulata* IFO 0720, No. 325, its mutant, No. OM-102, and *Saccharomycopsis lipolytica* IFO 1659 were also used.

Media and culture conditions. The seed culture medium contained 5% glucose, 0.3% NH₄Cl, 0.05% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.05% yeast extract (Difco) and 2% CaCO₃ in tap water. The basal fermentation medium contained 8% (v/v) *n*-tetradecane, 0.3% NH₄Cl, 0.05% KH₂PO₄, 0.05% KCl, 0.02% MgSO₄·7H₂O, 0.05% yeast extract (Difco), 20 µg per liter thiamine-HCl, 20 µg per liter biotin, 0.02% Emulgen 903 (a polyoxyethylene nonyl phenyl ether) and 5% CaCO₃ (a guaranteed reagent, sterilized separately) in tap water. The carbon sources and surfactants were changed, as described in the text and legends.

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Seed cultures were prepared by inoculating loopfuls of cells grown on potato-glucose agar slants into 300-ml Erlenmeyer flasks containing 40 ml of the seed culture medium, followed by incubation at 30°C for 2 days on a rotary shaker (220 rpm). The seed cultures (4 ml) were transferred to 500 ml Erlenmeyer flasks containing 50 ml of the basal fermentation medium. The flasks were shaken at 30°C for 6 days. A Waldhof type jar fermentor (Marubishi model MD-300, total volume, 5 l) was also used; incubation temperature, 30°C, agitation, 800 rpm, and aeration, 0.25 v/v/min. A seed culture (180 ml) was inoculated into the jar fermentor containing 3 l of the basal fermentation medium, from which CaCO₃ was omitted, and the pH was automatically maintained at 5.5 with 6 N KOH.

Analytical methods. Isocitric and citric acids were enzymatically assayed as described previously.¹¹ The remaining *n*-alkanes were determined by gas chromatography on a glass column (2 m) coated with Silicon OV-1 after extraction with *n*-hexane from the culture broth. Cell growth was determined as the optical density at 580 nm with a Shimadzu-Bausch & Lomb spectronic 20 colorimeter. The optical density of the culture broth containing the remaining *n*-alkanes was measured after the following treatment. One part of the culture broth was combined with two parts of a solvent system composed of ethanol, butanol and chloroform (10:10:1, v/v). The mixture was vigorously shaken and then centrifuged at 1450 × *g* for 10 min. To the culture broth, which still contained CaCO₃, an equal volume of 1.5 N HCl was added to dissolve the remaining CaCO₃.

The apparent affinity of cells for *n*-tetradecane was determined by the following methods. Cells were cultured at 30°C for 3 days on potato-glucose agar slants or on *n*-tetradecane agar plates, for which the basal fermentation medium was used and filter paper soaked in *n*-tetradecane was stuck on the lids of petri-dishes. Grown cells were suspended in 8 ml of 0.05 M phosphate buffer, pH 6.0. The optical density of the cell suspension was measured at 580 nm and defined as OD₁. *n*-Tetradecane (2 ml) was added to the cell suspension and the mixture was mixed well with a vibrator. After the mixture had been allowed to stand and the aqueous and *n*-tetradecane layers had been separated, the optical density of the aqueous layer was measured at 580 nm and defined as OD₂. The affinity of the cells for *n*-tetradecane was expressed as (OD₁-OD₂)/OD₁; a value of 1.0 represents the highest affinity of cells for *n*-tetradecane.

Chemicals. The *n*-alkane mixture was a gift from Nikko Petrochemical Co. The composition of the mixture was: C₁₀, 0.2; C₁₁, 1.5; C₁₂, 8.1; C₁₃, 46.9; C₁₄, 42.4; and C₁₅, 0.5%. Nissan Nonions and Emulgens were obtained from Nippon Oil & Fats Co. and Kao Soap. Co., respectively; their chemical structures are shown in Table II. All other chemicals were obtained from usual commercial

sources.

RESULTS

Acid productivity of different strains of C. catenulata from water-insoluble carbon sources

C. catenulata CBS 565, the type strain, and IFO 0720 were found to produce little isocitric acid, even from glucose. The other six strains, which had been classified as *C. ravauii*, were reported to be good producers of isocitric acid from glucose.¹¹ Their acid productivities were examined with four water-insoluble carbon sources, at a concentration of 6% (v/v), in the basal fermentation medium. As Table I shows, all the six tested strains produced isocitric acid in detectable amounts from some of the water-insoluble carbon sources, but there was less than 0.1 mg per ml of citric acid, a by-product, in all the culture broths in Table I.

Soybean oil seemed to be unsuitable for this fermentation. The previous type strain of *C. ravauii*,²¹ CBS 1904, was the best producer of isocitric acid, especially from *n*-alkanes, and was used mainly unless otherwise indicated.

Comparison of the affinities for n-tetradecane of cells of different strains

Preliminary experiments showed that the addition of some surfactants led to better growth of all tested strains of *C. catenulata* on water-insoluble carbon sources.

TABLE I. ACID PRODUCTIVITY OF DIFFERENT STRAINS OF *Candida catenulata* WITH NONCARBOHYDRATE OF CARBON SOURCES

Strain No.	Isocitric acid (mg/ml)			
	Carbon source			
	Soybean oil	Oleic acid	Tri-decane	Tetra-decane
CBS 1904	0.8	12.5	15.9	16.5
CBS 2015	2.6	0.2	0.8	0.1
CBS 2891	0.2	0.1	0.1	0.4
CBS 6174	0.2	0.1	12.9	12.5
No. 325	0.5	9.1	13	14.5
No. OM-102	0.5	11.9	1	14.8

The affinities of the cell-surfaces of different strains for water-insoluble *n*-tetradecane were measured. As a result, the affinities of the cell-surfaces of the four strains of *C. catenulata* CBS1904, CBS 6174, No. 325 and No. OM-102, were found to be one-fourth to one-third of that of *S. lipolytica* IFO 1659, a well-known *n*-alkane-assimilating yeast with a typical lipophilic cell surface. No significant differences in the affinities were observed between glucose- and *n*-tetradecane-grown cells of every strain.

Effects of the addition of different surfactants on acid production

Because of the hydrophilic cell surfaces of the *C. catenulata* strains and of their weak growth on the media containing water-insoluble carbon sources, it was necessary to find a suitable surfactant as an emulsifier for the carbon sources for cell growth and acid production. Table II shows the effects of the addition of different surfactants on cell growth and acid production by CBS 1904. The only markedly stimulatory surfactants were found to be Nissan Nonion NS-204.5, and Emulgens 903 and 905. Similar results also were observed for the other two strains, CBS 6174 and No. 325. Emulgen 903 was used in the basal fermentation medium at the concentration of 0.02%; lower and higher concentrations of the surfactant resulted in slightly lower acid yields. The addition of these surfactants hardly affected the acid production in glucose media.

Acid production from different n-alkanes

Figure 1A shows that CBS 1904 was able to use all the tested *n*-alkanes, ranging from C₁₀ to C₁₇, as carbon sources. The *n*-alkanes ranging from C₁₀ to C₁₄ gave better results as to cell growth and acid production, but the longer *n*-alkanes, C₁₅ to C₁₇, gave poor results. An *n*-alkane mixture, containing C₁₃ and C₁₄ mainly, gave similar good results as to cell growth and acid production.

Figure 1B demonstrates that No. OM-102 showed poor growth and little acid production

TABLE II. EFFECT OF THE ADDITION OF DIFFERENT SURFACTANTS ON CELL GROWTH AND ACID PRODUCTION

Strain CBS 1904 was cultured in the basal fermentation medium. All surfactants were added at a concentration of 0.02%.

Surfactant ^a	HLB ^b value	Growth (OD ₅₈₀)	Isocitric acid (mg/ml)
No addition		5	1.5
Span 85 (S-trioleate)	3.7	3	0.8
Span 80 (S-monooleate)	5.1	12	4.2
Span 20 (S-monolaurate)	8.0	10	3.8
Tween 85 (POES-trioleate)	11	12	5.0
Tween 60 (POES-monostearate)	14.9	No growth	
Tween 40 (POES-monopalmitate)	16	No growth	
NN S-4 (POE-stearate)	11.6	10	4.8
NN L-4 (POE-laurate)	13.1	8	3.8
NN S-40 (POE-stearate)	18.2	7	3.5
NN K-203 (POE-lauryl ether)	8.0	8	1.0
NN K-204 (POE-lauryl ether)	9.2	7	0.5
NN E-215 (POE-oleyl ether)	14.2	No growth	
NN NS-202 (POE-nonyl phenyl ether)	5.7	5	1.2
NN NS-204.5 (POE-nonyl phenyl ether)	9.5	30	22.0
NN NS-208.5 (POE-nonyl phenyl ether)	12.6	8	2.6
NN NS-215 (POE-nonyl phenyl ether)	15.0	3	1.0
Emulgen 903 (POE-nonyl phenyl ether)	7.8	28	22.5
Emulgen 905 (POE-nonyl phenyl ether)	9.2	28	21.4
Emulgen 909 (POE-nonyl phenyl ether)	12.4	8	2.4
Triton X-100 (POE-octyl phenyl ether)	13.5	12	2.0

^a NN, Nissan nonion; S, sorbitan; POE, polyoxyethylene; POES, polyoxethylene sorbitan.

^b Hydrophilic-lipophilic balance.

FIG. 1. Production of Isocitric Acid by Strains CBS 1904 (A) and OM-102 (B) from Different *n*-Alkanes.

□, isocitric acid; ■, growth.

not only on *n*-tridecane, as shown in Table I, but also on other odd-numbered *n*-alkanes, namely, undecane, pentadecane and heptadecane. This strain, however, gave good results on even-numbered *n*-alkanes, dodecane and tetradecane. An *n*-alkane mixture containing *n*-tridecane (46.9%) also gave similar poor results.

Effect of aeration

Different volumes of the basal fermentation medium were put into 500-ml Erlenmeyer flasks to examine the effect of aeration on acid production. As Fig. 2 shows, volumes of the medium between 25 and 50 ml caused little change in the acid production, but larger volumes caused marked decreases in acid production and cell growth.

Filamentous cells, similarly to in the static culture of No. OM-102 on glucose media,¹¹ were microscopically observed in the culture broth with the largest volume tested, 100 ml (Fig. 2).

Time course of acid production in a Waldhof type jar fermentor

In experiments with a Waldhof type jar fermentor, foaming was sometimes serious, and the conditions described under MATERIALS

FIG. 2. Effect of Aeration on Acid Production.

The indicated volumes of the basal fermentation medium were put into 500-ml Erlenmeyer flasks. ○, isocitric acid; ●, growth.

FIG. 3. Time Course of Isocitric Acid Production in a 5-liter Jar Fermentor.

○, isocitric acid; ●, growth; □, *n*-tetradecane.

AND METHODS gave good results. Figure 3 shows a typical time course of acid production under the used conditions. The fermentation was apparently growth-associated. After incubation for 80 hr, isocitric acid was excreted at a concentration of about 55 mg per ml; this value corresponded to an about 90% yield on a weight basis as to *n*-tetradecane supplied.

Recovery of isocitric acid from the culture broth

Culture broth (2 l), obtained with the jar fermentor and enzymatically determined to contain 110 g of isocitric acid, was filtered through Celite to remove the yeast cells. The filtrate was concentrated under reduced pres-

sure to about 400 ml. The concentrate was acidified with 6 N HCl to pH 3.50 with stirring and then left overnight in a refrigerator.⁴⁾ Colorless crystals, long prisms, were separated on a glass filter, washed with acetone and ether, and then dried at room temperature. The dried crystals (mp 182°C, neutral equivalent: found 115, calcd. for 115 as $\text{KH}_2\text{C}_6\text{H}_5\text{O}_7$) were enzymatically proved to be isocitrate, and weighed 118 g; the latter corresponded to 98.5 g of isocitric acid.

DISCUSSION

In the present study, CBS 1904, the previous type strain of *C. rautii*, was selected as the best producer of isocitric acid from an *n*-alkane mixture. No. OM-102, selected as the best producer of the acid from glucose in the previous study,¹⁾ definitely showed poor assimilability of odd-numbered *n*-alkanes (Fig. 1B). Unexpectedly, these two tested strains showed poor assimilability of *n*-alkanes of C_{15} and above (Fig. 1), as compared with other alkane-utilizing yeasts, but the *n*-alkane mixture used was mainly composed of C_{12} to C_{14} . Soybean oil, as a representative vegetable oil, and oleic acid, a liquid free acid, were poor as raw materials, probably owing to the carbon lengths of their fatty acids.

C. catenulata in the third edition of The Yeasts³⁾ seemed to consist of two strain groups, one of producers, the previous *C. rautii*,²⁾ and the other of non-producers of isocitric acid. The effects of the addition of surfactants as emulsifiers of *n*-alkanes on cell growth, in general, are reported in the literature to be inhibitory, neutral or stimulatory,^{5~7)} on account of differences in the chemical structures of the surfactants and in the affinities of the cell surfaces of different yeasts for *n*-alkanes. The surface-chemical functions of surfactants are often discussed in terms of their hydrophilic-lipophilic balance (HLB) values. Some *n*-alkane-assimilating yeasts, with strongly lipophilic cell-surfaces, can grow well on *n*-alkane media without the addition of any surfactant, and

were reported to show depressed cell growth on the addition of hydrophilic surfactants, with HLB values of 13 over, irrespective of their chemical structures.⁶⁾ Several yeasts, on the other hand, were reported to tend to show stimulated growth on *n*-alkane media on the addition of surfactants, with HLB values of 11 to 15.⁷⁾

The addition of lipophilic Emulgen 903 or 905, or Nissan Nonion NS-204.5 was essential for the acid production (Table II). They are polyoxyethylene nonyl phenyl ethers containing a biologically strange phenyl residue and have HLB values of 7.8 to 9.5. Other nonyl phenyl ethers showed no stimulatory effect on acid production. Other surfactants with similar HLB values also had no stimulatory effect. Accordingly, the essential function of Emulgen 903 *etc.* in acid production might be ascribable not only to emulsification of *n*-alkanes but also to an unknown biochemical interaction between the surfactants and the cell surfaces of *C. catenulata*.

The higher aeration in a shake culture gave better results as to acid production (Fig. 2). Furthermore, the use of a jar fermentor markedly improved the acid production (Fig. 3). This improvement was probably due to increases in the uptake rates of oxygen and, especially, *n*-alkanes due to the agitation in the fermentor in comparison with in the shaker.

Isocitric acid is usually produced simultaneously with citric acid by other species of yeasts.⁸⁾ Complications in product recovery, therefore, would arise. The culture broth of CBS 1904 contained no detectable amounts of polyols or organic acids other than isocitric acid, as judged on paper chromatography. Since *n*-alkanes were used as carbon sources in the initial fermentation medium, no water-soluble impurities originating from raw materials such as molasses were contained in the initial medium. Consequently, isocitric acid could easily be recovered as crystals of its monopotassium salt from the culture broth.

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