Kor. J. Appl. Microbiol. Bioeng.
Vol. 13, No. 3, 179-184 (1985)

Citrate Production by Sexually Compatible Strains of *Saccharomycopsis lipolytica*

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(Received June 1, 1985)

*Saccharomycopsis lipolytica*의 性的 和合性 菌体에 의한 시트르산 生産

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(1985년 6월 1일 수리)

Sexually compatible heterothallic haploids and diploids therefrom of *Saccharomycopsis lipolytica* were compared with respect to citrate production. Diploids constructed through mating were confirmed by random spore analysis, whereas those constructed through protoplast fusion were confirmed by haploidization. ATCC 44601 and IFO 1209 produced larger amount of citrate and isocitrate than IFO 1550 and IFO 1551. A mode of citrate production by diploids was intermediate of the parental haploid strains. The specific activities of citrate synthase and isocitrate lyase in IFO 1550 and IFO 1551 were higher than those in ATCC 44601 and IFO 1209, indicating little correlation between citrate production and specific activities of these enzymes.

The discovery of an ascogenous sexual cycle in *Candida lipolytica* by Wickerham *et al.* opened the way to analyse genetically this alkane yeast[1,2]. Zygote formation took place between two haploid cells with opposite mating types, designated A and B[3]. Although initial genetic studies were hampered by low frequency of mating and by low viability of ascospores, almost isogenic strains suitable for tetrad analysis have been constructed recently by successive inbreeding[4-6]. These strains of *Saccharomycopsis lipolytica* (formerly *Candida lipolytica*) were used for genetic analyses of alkane utilization[7] and extracellular protease production[8], which were unique properties not found in *Saccharomyces* or *Schizosaccharomyces* yeasts.

Apart from the sexual cycle, *Saccharomycopsis lipolytica* could be analysed genetically via parasexual cycle, i.e. protoplast fusion and mitotic haploidization[9,10]. In this study, citrate production by four haploid strains and diploids constructed by mating or by protoplast fusion was examined in order to know whether the production of citrate and isocitrate might be affected by genetic crosses of different strains.

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Materials and Methods

Strains

The property and origin of four wild-type strains are shown in Table 1. IFO 1550 and IFO 1551 are progenies of ascospores from a diploid strain, YB 423 isolated by Wickerham *et al.*[1]. ATCC 44601 was previously designated MT4[11]. Yeast cells were stocked on YMS slant as described by Oridziak *et al.*[4]

Media

The composition of minimal medium (MM) and complete medium (CM) are the same as described elsewhere[11]. Yeast extract/malt extract (YM) and restrictive growth (RG) media were prepared according to Ogridziak *et al.*[4]. V-8 sporula-

tion medium was prepared as follows; to V-8 juice (Campbell) 20 g/l of dry yeast (Oriental Yeast) was added, and pH was adjusted at 6.8 by KOH. The suspension was heated in boiling water bath for 10 min, filtered, and solidified by 2% agar. Production media contained 50 g glucose or 50 ml *n*-hexadecane as carbon source, 1 g ammonium sulphate, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g yeast extract, 1 mg $FeSO_4 \cdot 7H_2O$, 2 mg $MnSO_4 \cdot 4H_2O$, 2 mg $ZnSO_4 \cdot 7H_2O$, 0.05 mg $CuSO_4 \cdot 5H_2O$ per 1 liter of deionized water. *p*-Fluorophenylalanine medium was composed of 0.5% yeast extract, 2% glucose, 2 mM *p*-fluoro-DL-phenylalane, and 2% agar.

Mutagenesis

Auxotrophic mutants were induced by irradiation of ultraviolet light; cells grown in liquid CM overnight were suspended in sterile water and exposed to 254 nm light. The survival ratio was about 10%. Cells were then divided into small test tubes containing 1 ml CM, and grown. Auxotrophic cells were enriched in the MM by nystatin treatment as described by Snow[12].

Mating and random spore analysis

The procedures were essentially the same as published methods[4,5]. Briefly, haploid cells grown on YM plate were transferred onto RG plate and incubated for 2 days at 30°C. Cells were then mixed on fresh RG plate and incubated for another 2 days. The mixed cells were smeared on MM plate, where only diploid cells could grow, because initial haploid strains were marked with complementary auxotrophic mutations. The growing colonies on MM plate were picked and purified on MM, and tested for sporulation or by haploidization.

Sporulation was done on V-8 medium at 23°C. Heavy streaks were made on V-8 plate and incubated for at least 7 days. When asci formation was visible under a microscope, mycelium clumps were picked by sterile tweezers, and washed in sterile water to remove most of yeast-like cells. Asci attached to mycelia were then incubated in sterile water containing β-glucuronidase from Helix pomatia (Sigma). The suspension was sonicated in ice for 30 sec at 19.5 kHz and mixed with sterile paraffin oil in 1:1 volumetric ratio. The whole suspension was agitated and paraffin layer containing ascospores was plated on CM plate.

Protoplast fusion and haploidization

The preparation and fusion of protoplasts were conducted as described previously[10]. Fusion products were obtained on MM plate containing 0.6 M KCl and examined by haploidization whether they could produce parental or recombinant auxotrophic segregants. The randomly selected prototrophic clonies were grown on MM plate and transferred onto *p*-fluorophenylalanine plate. After incubation for 2 days at 30°C, cells were scraped off and inoculated into 1 ml CM and shaken to full growth. Cells were plated on CM, and auxotrophic colonies were detected by replica-plating onto MM. When the recovery of auxotrophic mutants was rare, nystatin enrichment was employed before plating onto CM.

Table 1. ***Saccharomycopsis lipolytica*** **strains**

strain	origin	mating-type	morphology
IFO 1209	NRRL, Y-1094 (L. J. Wickerham)	A	yeast-like
IFO 1550	NRRL, YB-423-3 (L. J. Wickerham)	A	mycelium
IFO 1551	NRRL, YB-423-12 (L. J. Wickerham)	B	mycelium
ATCC 44601	MT 4 (our stock)	B	yeast-like

Cultivation and chemical analyses

For citrate production, 100 ml production medium in 500 ml sakaguchi flask was shaken at 30°C. Cultivation was started by inoculating preculture grown overnight in 1 ml CM into glucose production medium, whereas preculture for *n*-hexadecane production medium was grown in 1 ml medium containing 2% (v/v) *n*-hexadecane, 2% peptone and 1% yeast extract. When pH of the culture decreased to about 4, 1 g dry-sterile $CaCO_3$ was added.

Cell density was measured by OD_{660} after dilution. Culture broth was firstly diluted twice with 1 N HCl to dissolve any residual $CaCO_3$. Glucose-grown cells were then diluted with water, whereas *n*-hexadecane-grown cells were diluted with solvent mixture containing *n*-butanol, ethanol and chloroform (10:10:1 in volumetric ratio). Acidified culture broth was centrifuged at 4,000 × g for 10 min, and the supernatant was filtered through glass filter paper (GS25, Toyo Roshi). Using the supernatant, citric acid and *threo*-Ds-isocitric acid were assayed by pentabromoacetone method and isocitrate dehydrogenase method, respectively[13].

Enzyme assays

Cell mass was prepared in the same production medium, except that 2 days' old glucose-grown cells and 3 days' old *n*-hexadecane-grown cells were harvested by centrifugation. Cells were washed by water and finally suspended in 50 mM imidazole-HCl buffer (pH 7) containing 12.5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol and 3% (v/v) glycerol. The cell suspension was disrupted mechanically through French press at 500 kg/cm2. The slurry was centrifuged at 27,000 × g for 15 min at 4°C. The supernatant was used for assays of citrate synthase (EC 4.1.3.7) accor-

ding to Srere *et al.*[14] and of isocitrate lyase (EC 4.1.3.1) according to Dixon and Kornberg[15]. Specific activity was expressed in milli units per mg of protein. One unit corresponds to one micro mole of product formed per min at room temperature. Protein was determined by Folin-Lowry method[16] using bovine serum albumin as the standard.

Results and Disscussion

Mating and random-spore analysis

In order to check the mating types of four wild-type strains listed in Table 1, each strain was converted to auxotrophic mutant. Mutant strains with complement auxotrophic markers were used for mass-mating. Table 2A and B shows the formation of prototrophic colonies from mixed cells on MM plate. Abundant prototrophic colonies grew from the mixture of IFO 1550 and IFO 1551. Because different auxotrophic derivatives of the same strain could not form prototrophs when mixed, mating is heterothallic as revealed by previous workers[1,2]. Mating types of IFO 1550 and IFO 1551 were designated as A and B, respectively according to the nomenclature of the predecessors[3,4]. IFO 1209 produced a few prototrophs only when combined with IFO 1551 (Table 2A), thus the mating type of IFO 1209 is A. The same fact was reported by other workers[17]. On the other hand, ATCC 44601 might be B-mating type, since this strain and IFO 1550 formed prototrophic colonies (Table 2B). However, prototrophic colonies might be trivially haploid revertants. This was checked by random spore analysis.

When the purified prototrophs were incubated on V-8 medium, asci were visible in culture from the cross between IFO 1550 and IFO 1551 (Fig. 1). Random spores were plated, and among the auxotrophic progeny, 13 Ade$^-$, 3 Met$^-$ and 11 Ade$^-$ Met$^-$ colonies were detected. On the other hand, all of the prototrophs obtained from cross between IFO 1209 and IFO 1551 produced very few asci, and within these asci no ascospore was visible. Futhermore, none of the prototrophs obtained from cross between ATCC 44601 and IFO 1550 formed asci. Hence, random spore analysis could not be performed in these prototrophs, and the mating type of ATCC 44601 remained unconfirmed.

Table 2. Mating responces

(A)

Strain	IFO 1209 Arg$^-$	IFO 1550 Ade$^-$	IFO 1551 Lys$^-$
IFO 1209 Met$^-$	—	—	+
IFO 1550 Ilv$^-$	—	—	+ ++
IFO 1551 Met$^-$	+	+++	—

(B)

Strain	ATCC 44601 Lys$^-$
IFO 1209 Arg	
IFO 1550 Ade	+
IFO 1551 Met	

Fig. 1. Ascospores from diploid between IFO 1550 Ade$^-$ and IFO 1551 Met$^-$ cultivated on V-8 medium.

IFO 1550 and IFO 1551 were apt to form mycelium on YM, whereas IFO 1209 and ATCC 44601 usually grew in yeast-like cells. Thus, cell surface structure might be differnt in these two morphologically distinguishable strains. Smooth colony type was correlated with yeast-like cells as reported by other workers[4], and these strains had low mating capacity.

Protoplast fusion and haploidization

Protoplast fusion mediated polyethyleneglycol and calcium ion can construct diploid irrespective of the mating type. When protoplasts from two auxotrophic strains with complementary markers were fused, prototrophic regenerants were obtained at an average frequency of 0.4×10^5 (Table 3). The heterozygous state for the auxotrophic markers was con-

Table 3. Protoplast fusion and haploidization

Parental strains (phenotype)	Regenerant	Fusion frequency	Auxotrophic segregant	
			Phenotype	No.
IFO 1209 3-8 (Arg^-), ATCC 44601 MU 7 (Lys^-)	F1-1	0.5×10^{-5}	Arg^-	19
			Lys^-	53
			Arg^-Lys^-	36
IFO 1551 3-2 (Met^-), ATCC 44601 MU 7 (Lys^-)	F2-1	0.4×10^{-5}	Lys^-	100
IFO 1209 3-8 (Arg^-), IFO 1551 3-2 (Met^-)	F3-1	0.3×10^{-5}	Arg^-	50
IFO 1550 3-2 (Ade^-), IFO 1551 3-2 (Met^-)	D1-10		Ade^-	2
			Met^-	18
			Ade^-Met^-	2

firmed by haploidization. Regenerant F1-1 produced both parental and recombinant type auxotrophs, whereas F2-1 and F3-1 yielded only one parental type auxotroph. Table 3 also demonstrates that diploid strain, D1-10 obtained from massmating procedure could be haploidized by *p*-fluorophenylalanine. This fact is striking contrast to that reported by other workers[4]; they found that haploidization was difficult for inbred strains of *Saccharomycopsis lipolytica*.

Citrate production by haploids and fusion products

Fig. 2 shows the cell growth and the production of citrate and isocitrate from glucose as carbon source by haploid strains. In glucose medium, citrate production was much higher than isocitrate production as already established by many workers[18-20]. Comparing the total amount of acid pro-

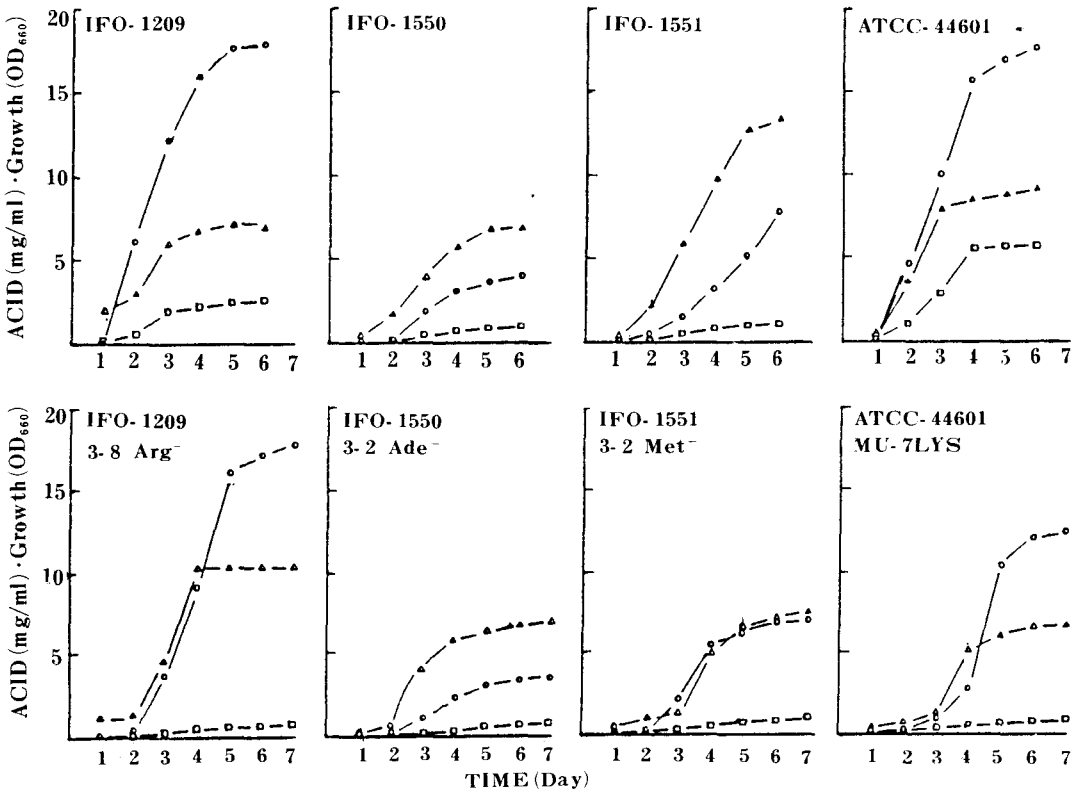

Fig. 2. Acid production in glucose medium. Symbols: citrate, —○—; isocitrate, —□—; OD_{660}, —△—

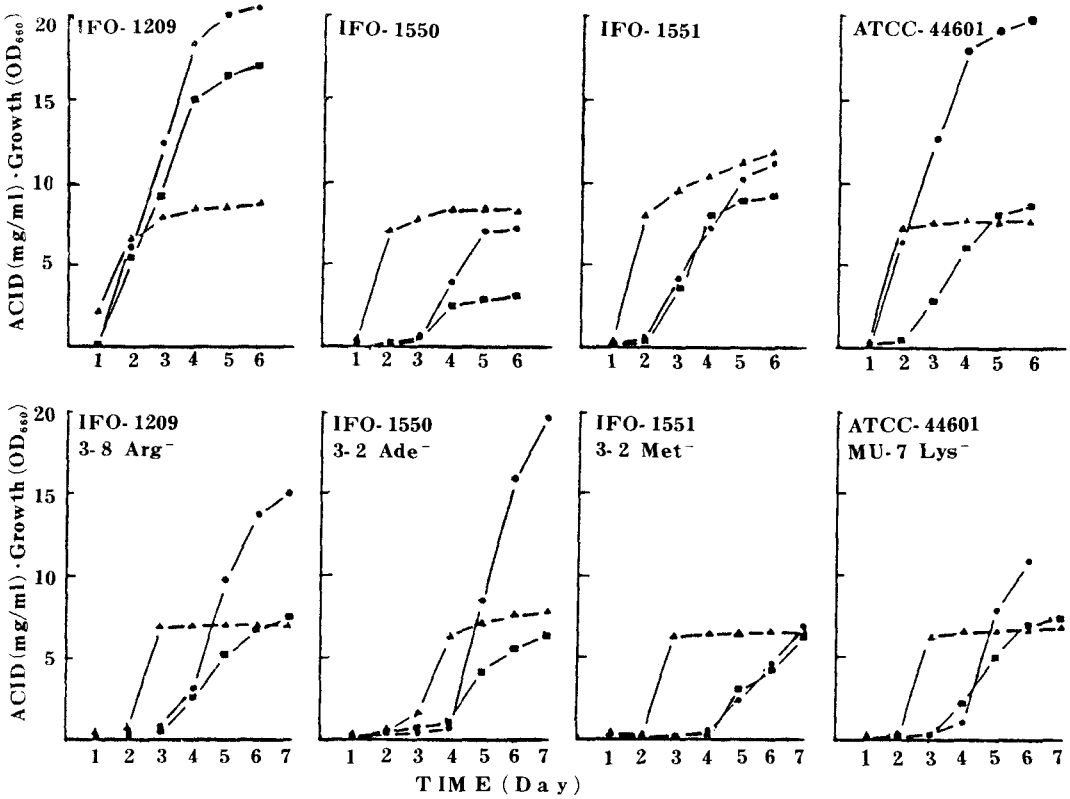

Fig. 3. Acid production in *n*-hexadecane medium. Symbols: citrate, —●—; isocitrate, —■—; OD_{660}, —▲—

duced, IFO 1209 and ATCC 44601 were high producers, while IFO 1550 and IFO 1551 were low producers.

The auxotrophic mutants derived from each haploid wildtype produced citrate and isocitrate similarly as the original strain when the culture medium was supplemented with amino acid or base requirements. Therefore, these mutant strains had not been impaired in production capacity.

Fig. 3 shows the same production time courses as Fig. 2 except that *n*-hexadecane was carbon source. In this medium, isocitrate production was considerably higher than in glucose medium. IFO 1550 and IFO 1551 again produced less amount of total acid than IFO 1209 and ATCC 44601.

Fig. 4 shows the production of acid by two fusion products, F1-1 obtained from IFO 1209 and ATCC 44601, and F2-1 obtained from IFO 1551 and ATCC 44601 (see Table 3). From only this figures, we could not obtain rigorous conclusion regarding the correlation between acid production capacity and genomic composition. However, F1-1, a fusion of two high producers seemed to accumulate higher amount of citrate and isocitrate than F2-1, a fusion of high and low producers. D1-10, a diploid from two low producers produced very little amount of citrate and isocitrate (data not shown). These results indicate that production of acid

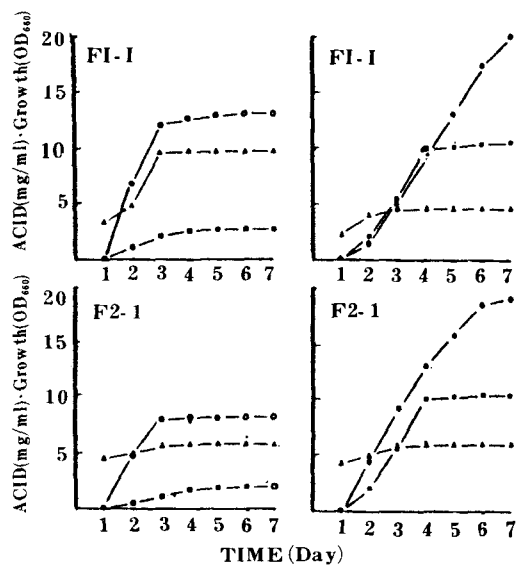

Fig. 4. Acid production in glucose medium (left, open symbols) and in *n*-hexadecane medium (right, closed symbols). Symbols: citrate (○, ●); isocitrate (□, ■); OD_{660} (△, ▲)

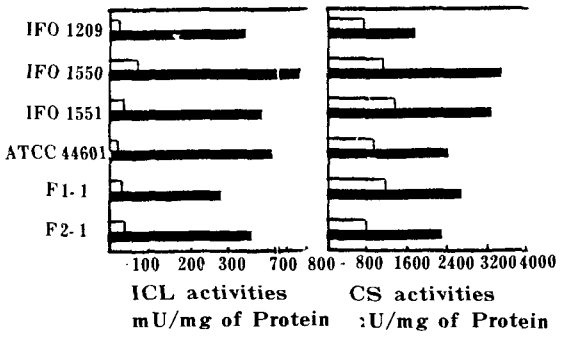

Fig. 5. Enzyme specific activities. ICL; isocitrate lyase (left), CS; citrate synthase (right). *Open bars represent activities in glucose-grown cells, while closed bars represent those in n-hexadecane-grown cells.*

depends upon genomic composition and that diploid produces intermediate amount of acid with compared to parental haploid strains. It may be possible that acid production is determined by many gene products and therfore no simple correlation exists between acid production and derivation of strains.

Enzyme activities in haploids and fusion products

Specific activities of citrate synthase and isocitrate lyase in glucose-grown and *n*-hexadecane-grown cells are shown in Fig. 5. Isocitrate lyase activity was in low level in glucose medium, since this enzyme is inducible only when the glyoxylate cycle is necessary for anaplerotic function[11]. Isocitrate lyase activity in IFO 1550 and IFO 1551 was higher than IFO 1209 and ATCC 44601 in *n*-hexadecane medium. Citrate synthase activity found in IFO 1550 and IFO 1551 was higher than those in IFO 1209 and ATCC 44601. These results were difficult to explain on the basis that two enzyme activities are important in citrate and isocitrate production. Namely, high production of acid by IFO 1209 and ATCC 44601 was not due to their higher activities of citrate synthase or isocitrate lyase. As for fusion products, F1-1 and F2-1, there was no evident conclusion as above. This fact suggests that acid production might not be related with intracellular level of key enzymes and might be related with cell surface permeability, possibly linked to cell morphology.

요 약

性的 和合性 heterothallic 2倍体와 1倍体인 *Saccharomycopsis lipolytica*에서 시트르산 生産性을 비교하였다. 交雜과 細胞融合에 의한 2倍体는 random spore分析과 單相化에 의하여 확인되었다. ATCC 44601과 IFO 1209 균주는 IFO 1550 와 IFO 1551 균주보다 시트르산과 이소시트르산을 많이 생산하였다. 2倍体에 의한 시트르산 生産양식은 両親 1倍体 菌株의 중간정도이였다. IFO 1550 와 IFO 1551 菌株의 citrate synthetase 와 isocitrate lyase의 比活性은 ATCC 44601과 IFO 1209 菌株에서 보다 높았고 이들 酵素의 比活性과 시트르산 生産間에는 相関関係가 없었다.

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