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Comparison of Different Strains of the Yeast *Yarrowia lipolytica* for Citric Acid Production from Glucose Hydrol

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ABSTRACT

Four commercial strains and two mutants of the yeast species *Yarrowia lipolytica* were screened using batch fermentation. Strain *Y. lipolytica* A-101-1.14 (induced with UV irradiation) was found to be the most suitable for citric acid production from glucose hydrol (39.9% glucose and 2.1% other sugars), a byproduct of glucose production from potato starch. The specific rate of total citric and isocitric acid production was 0.138 g/g·h, the yield on consumed glucose 0.93 g/g, and the productivity achieved was as high as 1.25 g/L·h. All of the tested yeast strains were able to utilize only the glucose from the glucose hydrol medium. Thus, some residual higher oligosaccharides remained in the process effluent.

Index Entries: *Yarrowia lipolytica;* citric acid fermentation; glucose hydrol; mutant; yeast.

INTRODUCTION

Various aspects of citric acid production from glucose by yeasts have been studied during the past three decades. According to kinetic investigations, the productivity and yield from glucose can be very promising.

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Moreover, considerably less isocitrate is excreted on glucose as compared to *n*-alkanes, the most common substrate for this process (*1–4*). In addition to typical batch fermentations, continuous cell-recycle systems (*5*) and immobilized-cell processes (*6–8*) have been developed. Despite significant knowledge of citric acid fermentation in synthetic glucose media, little information is available regarding the production of this compound from crude raw materials. Further, only citric acid fermentation of waste glucose (a residue from the recovery of fructose from sucrose hydrolysate) with *Candida oleophila* (*9*), and of molasses with *Candida guilliermondii* and *C. lipolytica* (*10*) or *Candida* sp. (*11*) has been described till now. In this paper we report the use of glucose hydrol for citric acid production by different strains of *Yarrowia* (before 1980, *Candida* or *Saccharomycopsis* [*12*]) *lipolytica*. Glucose hydrol is a byproduct of powdered glucose production from potato starch by a dual-enzyme process using α-amylase and glucoamylase. Three strains from the American Type Culture Collection, and *Y. lipolytica* A-101 and its two mutants have been compared for their product yields, specific growth, acid production rates, and ratios of citric acid to total citric and isocitric acids.

MATERIALS AND METHODS

Microorganism

Yarrowia lipolytica ATCC 8661, ATCC 20320, and ATCC 20324 from the American Type Culture Collection, and *Yarrowia lipolytica* A-101 isolated from soil in Poland and its two mutant strains, A-101-1.14 induced with UV irradiation and A-101-1.22 induced with NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) treatment, were maintained on yeast–malt–agar slants. The mutants, which had a survival rate of about 30%, were enriched by nystatin treatment (*13*), resulting in a 10^4 mutant-concentrating ratio. Of the survivors about 1% were desired mutants. The mutant A-101-1.14 did not grow on citrate; the mutant A-101-1.22 did not grow on acetate (unpublished results). Both mutants were obtained in the authors' laboratory in Poland.

Substrate

Glucose hydrol was obtained from Food Industry Concern, Lomza, Poland, where it occurs as a byproduct of glucose production from potato starch by a dual-enzyme (α-amylase and glucoamylase) process. The glucose hydrol contained 44.2% dry matter, 39.9% glucose, 2.1% other sugar (maltose, short-chain dextrins), 1% ash, 0.6% chlorides, 0.29% total nitrogen, and 0.5% phosphorus as P_2O_5.

Media

The growth medium of the inoculum contained 40 g glucose, 6 g ammonium chloride, 0.5 g potassium dihydrogen phosphate, 1 g magnesium sulfate, 1 g yeast extract, 0.2 mg thiamine-HCl, and 10 g calcium carbonate in 1 L tap water. *The acid-production medium* contained 400 mL glucose hydrol, 2 g ammonium chloride, 0.2 g potassium dihydrogen phosphate, 1 g magnesium sulfate, and 1 g yeast extract in 1 L tap water.

Culture Conditions

An inoculum of 300 mL was introduced into a stirred jar fermenter (AK-210, Vneshtechnica, Moscow, Soviet Union) containing 5.2 L of the production medium. Cultivation was carried out at 30°C. The pH was automatically controlled at 5.5 and adjusted with 10*N* sodium hydroxide. The agitation rate was 700 rpm, and the aeration rate was 0.35 V/V·min during the excretion phase and up to 0.7 V/V·min during active cell growth.

Analytical Methods

Biomass was determined by the dry weight method, glucose with a glucoseoxidase-peroxidase reaction kit (Fermognost, VEB Laborchemie Apolda, Apolda, Germany (East), citric acid by a pentabromoacetone method (*14*), and isocitric acid by an enzymatic method (*15*).

Calculation of Kinetic Parameters

The specific total acid production rate [q_P, (g acid)/(g cells)·h], the average biomass [$\bar{X}$], and the amount of acid [P] were calculated for each time interval as given in Figs. 1 and 2. The specific acid production rate was calculated according to the following equation (1):

$$q_P = \frac{d[P]/(dt)}{[\bar{X}]} \quad (1)$$

Table 1 shows the kinetic parameters for specific total acid [q_P, (g acid)/(g cells)·h], citric acid [q_{CA}, (g acid)/(g cells)·h], and isocitric acid [q_{ICA}, (g acid)/(g cells)·h] production, and glucose consumption [q_S, (g glucose)/(g cells)·h] rates during the stationary growth phase, and was calculated from smoothed curves. The yield of total acid [$Y_{P/S}$, (g acid)/(g glucose consumed)] was calculated in reference to the values of total acid and glucose consumed at the beginning and end of the stationary growth phase. In Table 2 the volumetric overall productivity [Q_P, (g acid)/L·h] and the yield (Y_{total}, (g acid)/(g glucose consumed)] were calculated in reference to the values between the beginning of the process and the point at which the total of citric and isocitric acids reached its maximum.

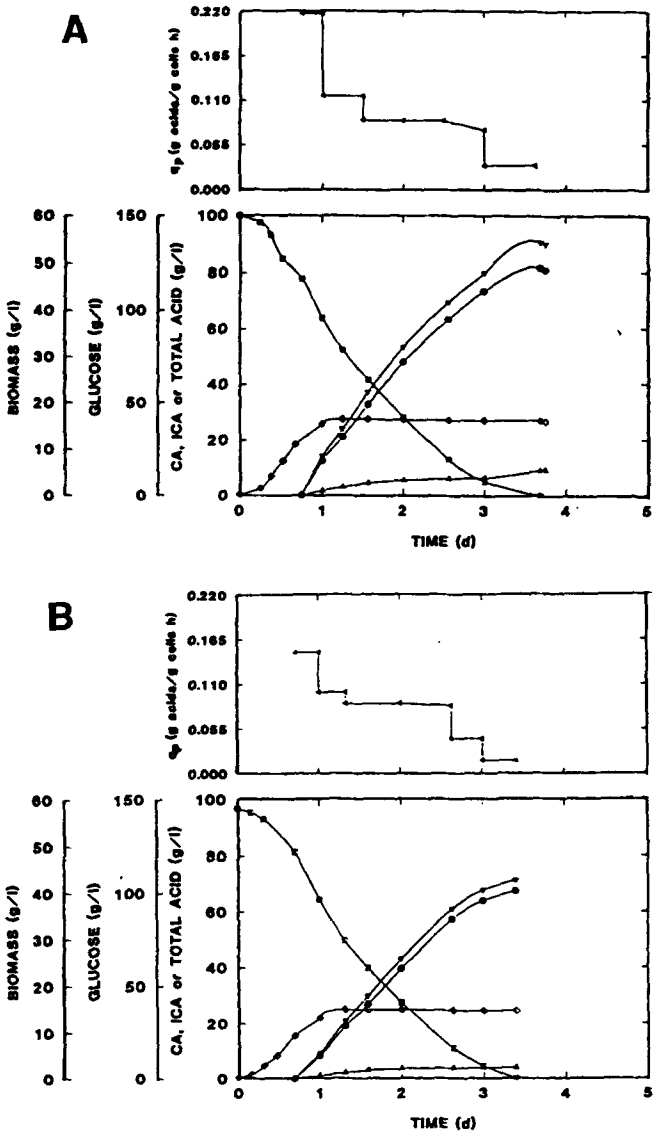

Fig. 1. Citric (●), isocitric (▲), and total (▼) acid; biomass (◇); glucose (□); and specific total citric acid production rate (◄) during batch cultivation of *Yarrowia lipolytica* ATCC 8661 (**A**), ATCC 20320 (**B**), and ATCC 20324 (**C**).

RESULTS AND DISCUSSION

Cell Growth and Citrate Production by Different Strains

For any fermentation process based on waste substrates, it is advantageous if the producing microorganism is capable of utilizing all of the sugars present in the substrate and shows little sensitivity to impurities in

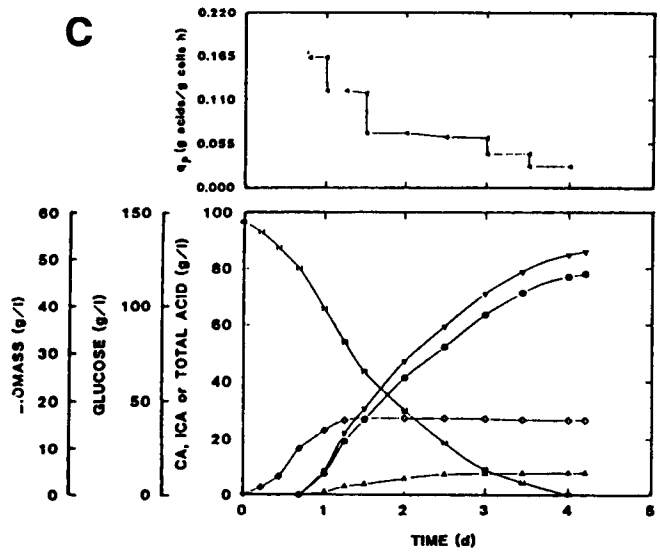

Fig. 1C.

the substrate. Glucose hydrol, containing 42% sugars, of which 39.9% was glucose and 2.1% maltose and short-chain dextrins, was used as the carbon source for citric acid production with *Yarrowia lipolytica* yeast. The earlier, unpublished studies by Wojtatowicz and Rymowicz showed that none of the numerous efficient producers of citric acid belonging to the species *Yarrowia lipolytica* could metabolize maltose and short-chain dextrins in glucose hydrol. Therefore, some residual sugar was expected to remain in the medium and for calculations we considered only glucose as the carbon source.

Six strains of *Yarrowia lipolytica* (ATCC 8661, ATCC 20320, ATCC 20324, A-101, A-101-1.14, and A-101-1.22) were screened for their ability to produce citric acid on glucose hydrol medium. The time-courses for growth, acid production, and glucose consumption for each yeast culture are shown in Figs. 1 and 2. Profiles of cell growth and acid production for four examined strains (ATCC 8661, ATCC 20320, and ATCC 20324 in Fig. 1, and A-101 in Fig. 2A) were very similar and corresponded to the general characteristics of citrate fermentation reported earlier (*1,4,16*). Yeast growth was found to proceed exponentially until extracellular nitrogen was completely consumed. A stationary growth phase then occurred. Citric and isocitric acid production started when the exponential growth ceased (about 15–18 h of cultivation). The highest specific total citric acid production rate (0.120–0.217 g/g·h) took place in the beginning of the production phase during a short period of 14–18 h only. The production rate decreased shortly after biomass had reached its maximum level at about 30 h. After 30 h, the production rate of citric acid proceeded at a markedly lower and quite constant level of about 0.06–0.085 g/g·h for the next 40–50 h. This is in agreement with the results of Behrens et al. (*2,17*), who have previously

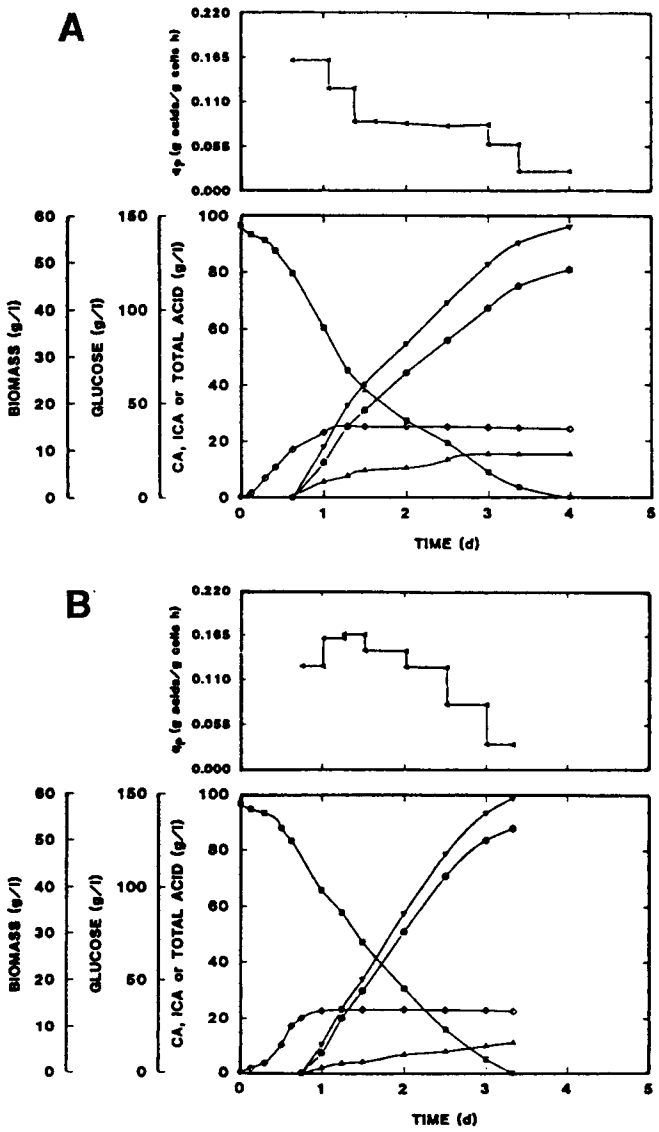

Fig. 2. Citric (●), isocitric (▲), and total (▼) acid; biomass (◇); glucose (□); and specific total citric acid production rate (◄) during batch cultivation of *Yarrowia lipolytica* A-101 (**A**), A-101-1.14 (**B**), and A-101-1.22 (**C**).

reported two distinctly different citric acid production phases with yeast with specific total citric acid production rates of 0.18–0.14 g/g·h and corresponding yields of 0.70–1.20 g/g.

The remaining two strains (A-101-1.14 and A-101-1.22) behaved differently (Fig. 2B and 2C). With the strain A-101-1.14 the specific total citric acid production rate was constant at about 0.138 g/g·h (Fig. 2B). The

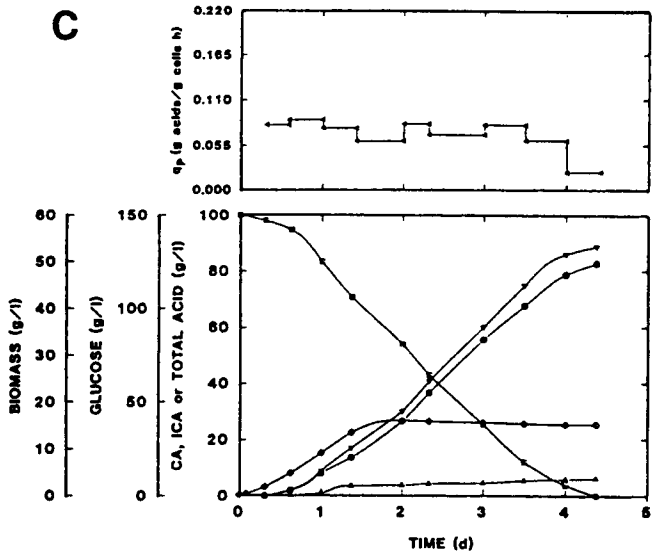

Fig. 2C.

strain A-101-1.22 also had a constant, but markedly lower, specific total citric acid production rate of 0.08 g/g·h (Fig. 2C). With A-101-1.22, citric acid production started parallel to the cell growth, even in the presence of some nitrogen. This confirms the observation of Moresi et al. (*18*) concerning the acid excretion of *Candida lipolytica* Cooper during the cultivation on a hydrocarbon medium. In the present work each process varied with the dynamics of cell growth and acid production, the amount of the formed biomass and the accumulated acids, and the ratios of citric to total citric and isocitric acid (Figs. 1 and 2). The biomass concentration stabilized on a certain level between 22 h of cultivation with A-101-1.14 and 40 h with A-101-1.22. Even though the initial nitrogen concentration was equal in each process, the final biomass concentration varied from 13.6 g/L with A-101-1.14 to about 16.5 g/L with ATCC 8661 and ATCC 20324.

The Effect of Mutation on Cultivation and Production Time

The cultivation time of the strain A-101 was shortened, by UV mutation, from 31 to 24 h for maximal biomass concentrations of 15.2 g/L and 14.1 g/L, respectively. The acid production time was also shortened from 96 to 80 h for maximal total citric acid concentrations of 96.2 g/L and 100 g/L, respectively. Strain A-101-1.22, obtained with NTG-mutation, had the longest cultivation time: 48 h for the maximal biomass concentration of 16 g/L and 105 h for the maximal total citric acid concentration of 88.7 g/L. With strains ATCC 20320 and A-101-1.22, the ratios of citric to total

Table 1
Kinetic Parameters of Citric Acid Fermentation on Glucose Hydrol by Various Strains of *Yarrowia lipolytica*

Strain	μ_{max}[a]	$Y_{X/S}$[b]	q_S[c]	q_P[d]	q_{CA}[d]	q_{ICA}[d]	$Y_{P/S}$[e]
A-101	0.22	0.42	0.091	0.084	0.071	0.013	0.92
A-101-1.14	0.24	0.42	0.148	0.138	0.121	0.017	0.93
A-101-1.22	0.09	-	0.112	0.081	0.077	0.004	0.72
ATCC 8661	0.21	0.41	0.108	0.085	0.077	0.008	0.79
ATCC 20320	0.18	0.37	0.126	0.085	0.080	0.005	0.67
ATCC 20324	0.17	0.36	0.075	0.061	0.055	0.006	0.81

[a] μ_{max} = maximum specific growth rate, 1/h.
[b] $Y_{X/S}$ = cell yield coefficient during the exponential phase, (g cells)/(g glucose).
[c] q_S = specific rate of glucose consumption during the stationary phase, (g glucose)/(g cells)·h.
[d] q_P, q_{CA}, q_{ICA} = specific production rates of total, citric, and isocitric acid, respectively, during the stationary phase, (g acid)/(g cells)·h.
[e] $Y_{P/S}$ = yield of total acid during the stationary phase, (g acid)/(g glucose consumed).

Table 2
Overall Productivity and Yield of Citric Acid Fermentation on Glucose Hydrol by Various Strains of *Yarrowia lipolytica*

	Strain					
Parameter	A-101	A-101 -1.14	A-101 -1.22	ATCC 8661	ATCC 20320	ATCC 20324
Q_P[a]	1.01	1.25	0.85	1.05	0.92	0.91
Y_{total}[b]	0.67	0.71	0.60	0.61	0.51	0.59

[a] Q_P = productivity of total citric and isocitric acid referred to the overall process, (g acid)/L·h.
[b] Y_{total} = yield of total citric and isocitric acid referred to the overall process, (g acid)/(g glucose consumed).

acid were 0.95 and 0.94, respectively, and with the fastest strain A-101-1.14, only 0.88. Strain ATCC 20320 reached a total citric acid concentration of 71.3 g/L in 82 h, the lowest maximal total citric acid among the investigated strains.

Kinetic Constants

Some of the characteristic fermentation parameters of the cultivations in Figs. 1 and 2 are shown in Table 1. The specific production and consumption rates are the rates obtained only during the acid production phase. The specific total acid production rate (q_P) was highest for the mutant strain A-101-1.14 (0.138 g/g·h). For the strains A-101, A-101-1.22, ATCC 8661, and ATCC 20320 the rate varied little, within the range of 0.081 to 0.085 g/g·h, and for the strain ATCC 20324 the rate was only 0.061 g/g·h. The yield ($Y_{P/S}$), based on glucose consumed, ranged from 0.67 g/g

with strain ATCC 20320 to 0.93 g/g with strain A-101-1.14. The yields of the total citric and isocitric acids are not based on glucose used for cell mass and maintenance during trophophase. The specific production rate and yield obtained with strain A-101-1.14 were higher than those values of 0.037–0.070 g/g·h (production rate) and 0.40–0.87 g/g (yield) reported earlier (*1,5,8,16*) in batch cultivations with yeast *Yarrowia lipolytica* grown on synthetic glucose media.

In addition to the volumetric productivity, Table 2 also shows the yield of total acid during the overall fermentation. The highest values (1.25 g/L·h and 0.71 g/g) of both parameters were obtained by the mutant strain A-101-1.14. The overall yields (Y_{total}) based on glucose consumed for both production and cell growth, shown in Table 2, are lower than the yields of total acid during the stationary phase ($Y_{P/S}$) in Table 1.

CONCLUSION

The six strains of *Yarrowia lipolytica*, ATCC 8661, ATCC 20320, and ATCC 20324, our own strain A-101, and its two mutants A-101-1.14 (with UV irradiation) and A-101-1.22 (with NTG treatment), were investigated in batch fermentation for citric acid production on glucose hydrol medium. The highest citric acid concentration, 100 g/L with the maximum biomass concentration of 14.1 g/L, was attained by the mutant strain A-101-1.14 following the shortest cultivation (24 h) and production (80 h) times. The highest overall volumetric productivity was 1.25 g/L·h and yield 0.93 (g acid)/(g glucose consumed). It was shown that the mutant (acetate−) strain A-101-1.22 was better in utilizing carbohydrate for citric acid production than the mutant (citrate−) strain A-101-1.14. It excreted less isocitrate (6 g/L compared to 11.6 g/L). Cell growth consumed a significant fraction of substrate at the expense of product. Overall yield could possibly be improved by using continuous cell-recycle or immobilized-cell systems. The results suggest that impurities in glucose hydrol did not affect citric acid production.

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