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MARIA WOJTATOWICZ
WALDEMAR RYMOWICZ

EFFECT OF INOCULUM ON KINETICS AND YIELD OF CITRIC ACIDS PRODUCTION ON GLUCOSE BY *Yarrowia Lipolytica* A-101

Department of Biotechnology and Food Microbiology, Academy of Agriculture, Wrocław

Key words: *Yarrowia lipolytica* A-101, citric and isocitric acid, glucose, nitrogen deficient medium

The effect of two different inocula on growth and production parameters in citric acid fermentation (on glucose) by *Yarrowia lipolytica* A-101 was studied. For inoculum prepared in full growth medium the total acids yield was 5-12% higher and biomass yield about 10% higher than for inoculum prepared in a nitrogen-deficient medium. The latter inoculum, however, led to about 10-30% higher acid production and glucose consumption rates.

Until the early 1970s practically the only organisms used to produce citric acid were *Aspergillus niger* and a few other fungi. Today we know that many kinds of yeasts can accumulate substantial amounts of citric acid in their growth media. The most efficient citric acid producers belong to the *Candida genus*, and the strains used most often are *C. lipolytica, C. zeylanoides, C. parapsilosis, C. tropicalis, C. guilliermondii, C. oleophila, C. petrophilum* and *C. intermedia* [10]. Yeasts can produce citric acid more rapidly than fungi and in a greater variety of substrates including n-alkanes, n-alkenes, glucose, molasses, acetate, alcohols, fatty acids and natural oils [4, 8, 9, 17, 18]. The product yield on n-alkanes and vegetable oils may be as high as 1.6 g/g [4, 9]; on glucose it is usually comparable to that in processes involving filamentous molds [4-6]. One disadvantage of yeasts is that they produce substantial amounts of isocitric acid, especially when noncarbohydrate C-sources are used. An improvement of the citric-to-isocitric acid ratio can be achieved by using certain mutants [1, 9] or by adding aconitase inhibitors to production media [13, 17].

Diverse aspects of the overproduction of citric acid by various yeast strains have been studied [2, 3, 5, 6]. So far there have been no reports on the effect of inoculum kind on parameters of kinetics and yield in yeast citric acid fermentation. We decided to look into this matter.

EXPERIMENTAL

MATERIAL AND METHODS

Microorganism. The yeast strain used in our research was *Yarrowia lipolytica* (previously described as *Candida* or *Saccharomycopsis*) A-101, isolated and identified at our laboratory.

Media and culture conditions. The media used for cell growth and citric acid production are described in Table 1. Seed cultures were maintained for two days at 28°C in two-liter Erlenmeyer flasks containing 250 ml of inoculation medium (A) or (B), placed on a shaker. The biomass was then separated and transferred to a 10-liter jar fermentor (AK-210 type, Vneshtekhnika, USSR) containing 5 liters of production medium. Cultivation was carried out at 30°C and pH 5.5, automatically adjusted with 10 N NaOH. Agitation speed was 700 rpm, aeration rate was 0.2 vvm during the acid production phase and up to 0.5 vvm during active yeast cell growth.

Table 1. Composition of seed and production media

Ingredient		Seed medium		Production medium
		(A)	(B)	
Glucose	(gl^{-1})	40	40	60*
NH_4Cl	(gl^{-1})	6	1.5	1.5
KH_2PO_4	(gl^{-1})	0.5	0.5	0.2
$MgSO_4 \times 7H_2O$	(gl^{-1})	0.5	0.5	1.0
$FeSO_4 \times 7H_2O$	(mgl^{-1})	2.0	2.0	2.0
$ZnSO_4 \times 7H_2O$	(mgl^{-1})	2.5	2.5	2.5
$MnSO_4 \times 1H_2O$	(mgl^{-1})	1.0	1.0	1.0
$CuSO_4 \times 5H_2O$	(mgl^{-1})	0.1	0.1	0.1
Thiamine — HCl	(mgl^{-1})	0.2	0.2	—
Yeast extract	(gl^{-1})	1.0	1.0	1.0
$CaCO_3$	(gl^{-1})	10	10	—

* initial glucose concentration

Glucose concentration at the start of fermentation was 60 gl^{-1}, and then four additions of 60% glucose solution of total volume of 400 ml were made. All changes in the volume of fermentation wort due to glucose additions, pH adjustement and samples removal were taken into account when calculating the balances.

Analytical methods. Biomass was determined by the dry weight method. 5-10 ml samples were filtered on Millipore HA filters, the biomass was washed in distilled water and dried to constant weight at 105°C. Intracellular nitrogen was determined by Kjeldahl's method, glucose concentration by Nelson's method

[14], citric acid by the pentabromacetone method [15], and isocitric acid by the enzymatic method [16], Both acids were expressed as monohydrates.

RESULTS AND DISCUSSION

Y. lipolytica yeasts overproduce citric and isocitric acids on glucose and other carbon sources in conditions of nitrogen deficiency. Generally, extracellular acids accumulation begins after the exponential growth stage, this being caused by the exhaustion of nitrogen in the medium, and becomes linear during the stationary phase [3, 5, 8, 11]. Specific rates of acids production, productivity and the ration between both secreted acids very depending on yeast strain, carbon source, culture conditions, and cultivation system [2, 6, 11-13, 17]. Seed cultures are prepared using either rich complex media [6] or synthetic media with a composition very similar to that of the production medium [12].

To determine whether the manner of inoculum preparation affects the basic parameters of citric fermentation on glucose by *Y. lipolytica* A-101 we analyzed

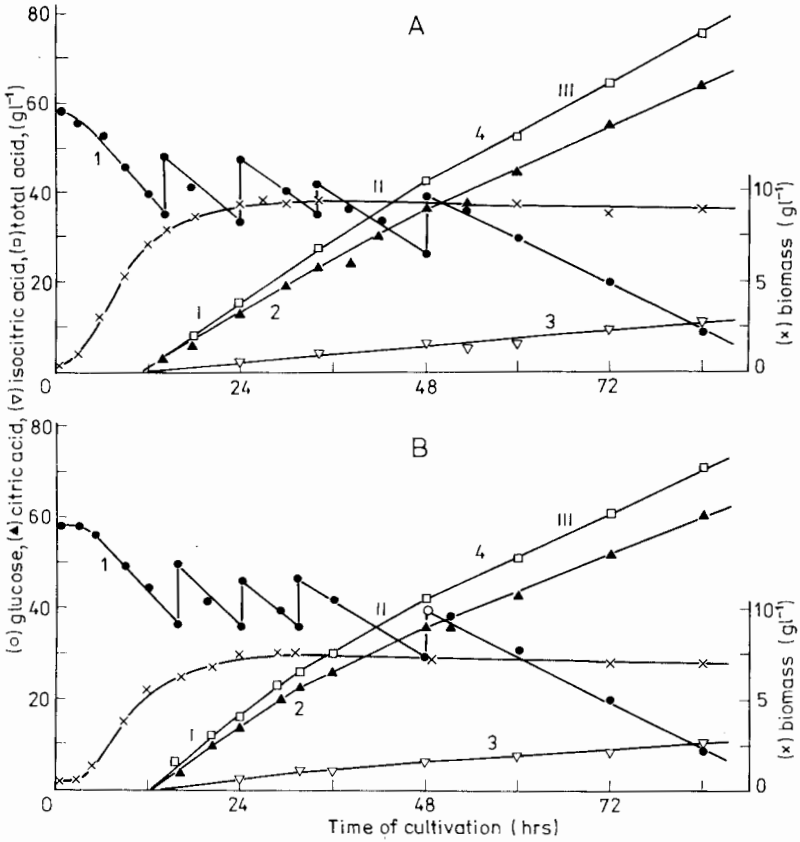

Fig. Citric and isocitric acid production on glucose in fedbatch cultures of *Y. lipolytica* A-101 at different inoculum; A — seed culture grown in full medium (C:N = 10): biomass — 13.1 gl^{-1}; cellular nitrogen content — 8.2%; no CA and ICA; B — seed culture grown in nitrogen-deficient medium (C:N = 40): biomass — 12.2 gl^{-1}; cellular nitrogen content — 4.0% and ICA — 5.5 gl^{-1}

two processes using different starting cultures. In process A the starting culture medium contained an optimum amount of nitrogen (C:N = 10), while process B was started with cells grown in a nitrogen-deficient medium (C:N = 40). Biomass in preculture A was slightly higher (13.1 gl^{-1}) than in preculture B (12.2 gl^{-1}) and its cells contained twice the amount of nitrogen (8.2%) than those cultured in nitrogen-deficient conditions (4.0%; cf. Fig.). Fermentation proper was carried out in stationary culture with feed supply at total glucose concentration of 104 gl^{-1} and growth adjusted to about 9-10 gl^{-1} by means of 1.5 gl^{-1} NH_4Cl.

Fig. shows curves for cell growth, glucose consumption, and citric and isocitric acids production revealing differences between the two processes that were studied. In fermentation A yeast growth began practically without any lag phase, while fermentation B had a lag phase of almost 4 h. Yeast growth was exponential until biomass content of about 5-5.5 gl^{-1} and then became linear with a greatly reduced rate. Maximum biomass level in proces A was 9.6 gl^{-1} after 27 h of culture, while in process B it was much lower 7.5 gl^{-1} after 24 h of fermentation.

As is known, yeast growth during the linear phase is sustained by intracellular nitrogen, given the lack of this element in the medium [5, 8]. Briffaud and Engasser [5] reported for example that nitrogen in *S. lipolytica* D1805 biomass decreased from 8.5 to 4%. Almost identical changes were observed in process A in our studies, but not in fermentation B in which cells retained more intracellular nitrogen (5.1%). This, together with the fact that the cells in the latter process contained less nitrogen at the outset and that biomass yield was lower than in the former process can account for the lower biomass production in fermentation B. Specific growth rates were similar in both processes (Table 2).

Tabele 2. Effect of inoculum of the growth parameters in citric acids production on glucose by *Y. lipolytica* A-101

Parameter	Unit	Definition	Values for inoculum: (A)	(B)
X_m	gl^{-1}	Maximum biomass	9.6	7.5
$N_{exp.}$	%	Cellular nitrogen content in exponential and stationary growth phase, respectively (g nitrogen/100 g biomass)	8.3	8.1
$N_{stat.}$	%		4.2	5.1
μ_m	h^{-1}	Specific growth rate during exponential phase	0.24	0.23
μ_l	h^{-1}	Specific growth rate during linear phase	0.024	0.024
$Y_{x/s}$	gg^{-1}	Biomass yield coefficient in exponential phase (g biomass / g glucose consumed)	0.38	0.34

Citric and isocitric acids production began about the 12th hour of cultivation (Fig.). The production rates were not constant during the entire excretion phase, contrary to most of the earlier reports [5, 8, 11, 12, 17, 18], with three periods of ever decreasing production rates being distinguishable. Similar phased citric acids production was reported by Behrens et al. [3, 4] for *S. lipolytica* EH 59 in fed-batch type cultures on glucose.

The values of basic fermentation parameters collected in Table 3 show that specific rates of glucose consumption and of acids production were clearly higher for the inoculum prepared in the nitrogen-deficient medium. On the other hand, the total acids yields were higher when the inoculum was grown in the full medium.

Table 3. Specific consumption and production rates, yield of total acid, and citric to combined citric and isocitric acid ratio in citric acid production on glucose by diffecent. *Y. lipolytica* A.101 inocula

Parameter	Unit	Definition	Values at inoculum:	
			(A)	(B)
q_s^I	h^{-1}	Specific rate of glucose consumption in successive	0.170	0.254
q_s^{II}	h^{-1}	stages (I, II, III) of production phase*	0.120	0.143
q_s^{III}	h^{-1}	(g glucose/g biomass x h)	0.092	0.116
q_p^I	h^{-1}	Specific rate of total acid production in successive	0.143	0.204
q_p^{II}	h^{-1}	stages (I, II, III) of production phase*	0.122	0.138
q_p^{III}	h^{-1}	(g total acid/g biomass x h)	0.103	0.115
$y_{p/s}^I$	gg^{-1}	Yield of total acid in successive (I, II, III)	0.84	0.80
$yy_{p/s}^{II}$	gg^{-1}	stages of production phase*	1.02	0.97
$y_{p/s}^{III}$	gg^{-1}	(g total acid/g glucose consumed)	1.12	0.99
$y_{p/s}^g$	gg^{-1}	Global yield of total acid (g total acid/g glucose consumed)	0.87	0.82
$\frac{CA}{CA+ICA}$	w/w	Citric to total citric and isocitric acid ratio	0.85	0.86

* successive stages of acid excretion are shown in Fig.

Specific production and consumption rates kept decreasing in the successive phases of acids production. These drops were less severe in culture A which suggests a greater stability of acids synthesis and excretion than in culture B. Nevertheless, even the lowest values of specific production rates in the third stage

of the excretion phase (0.103 h^{-1} and 0.115 h^{-1} for A and B respectively) were at least twice higher than in the case of other *Y. lipolytica* strains [5, 6, 11]. In contrast, the total acids yield was the lowest in the first stage of the production phase, to eventually rise to as much as 1.12 (process A) and 0.99 gg^{-1} (process B) in the third stage. The former value is close to the theoretical yield of monhydrate citric acid from glucose (1.17 gg^{-1}) when oxalacetate is anapletorically reproduced by pyruvate carboxylation [17]. However, Briffaud and Engasser [5] as well as Marchal et al. [11] found that this process does not supply sufficient subsistence energy during the production phase and that additional energy production in the Krebs cycle is necessary. This suggests that towards the end of the production cycle *Y. lipolytica* A-101 could have synthesized citrate using intracellular reserve substances or previously formed polyols. The latter products may be accumulated in citric acid producing yeast cultures under various conditions [7, 17].

The figures in Table 3 also show that the ratio of citric acid to combined citric and isocitric acids was roughly the same for both kinds of inoculum — 0.85 and 0.86 (w/w).

CONCLUSIONS

1. The inoculum cultivated in full growth medium (A) produced ca. 5-12% more total acids than cells grown in nitrogen-deficient medium (B); also, the synthesis and excretion of acids was more stable for the former medium, and growth yield and biomass figures were 10% higher.

2. Specific rates of acids production and glucose consumption were 10-30% higher for inoculum B than for inoculum A.

3. The growth of yeast A commenced immediately after their transference to the production medium, while in the case of yeast B there was a ca. 4-h lag phase.

4. The kind of inoculum had practically no effect on the citric acid-to-combined citric and isocitric acids ratio, and on the specific growth rate of *Y. lipolytica*.

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M. Wojtatowicz, W. Rymowicz

WPŁYW INOKULUM NA KINETYKĘ I WYDAJNOŚĆ PRODUKCJI KWASÓW CYTRYNOWYCH NA GLUKOZIE PRZY UŻYCIU *Y. LIPOLYTICA* A-101

Katedra Biotechnologii i Mikrobiologii Żywności, Akademia Rolnicza, Wrocław

Streszczenie

Oceniano wpływ inokulum na wydajność oraz kinetykę wzrostu i produkcji kwasów cytrynowych na glukozie przy użyciu *Y. lipolytica* A-101. Przeprowadzono 2 hodowle produkcyjne typu fed-batch, pierwszą (A) szczepiono komórkami wyrosłymi w pełnym podłożu wzrostowym, przy C:N = 10, drugą (B) komórkami wyrosłymi w podłożu z deficytem azotu, przy C:N = 40. Kultura inokulacyjna (B) różniła się od (A) nieco niższą biomasą, 2-krotnie niższą zawartością azotu komórkowego i rozpoczęciem fazy produkcji kwasów. Charakter wzrostu, konsumpcji glukozy oraz produkcji kwasów był zbliżony w obu hodowlach, z wyjątkiem, że w pierwszej (A) drożdże rozpoczynały wzrost prawie natychmiast po wprowadzeniu do podłoża produkcyjnego, zaś w (B) lag-faza trwała blisko 4 h. Produkcja kwasów rozpoczynała się w przybliżeniu ok. 12 h hodowli i jej szybkość nie była stała w ciągu całego procesu; można było wyróżnić 3 stadia o malejącym tempie ekskrecji kwasów. Następujące różnice w wydajnościach, tak wzrostu jak i produkcji kwasów ogółem oraz we właściwych szybkościach produkcji i konsumpcji były stwierdzane: w procesie (A) wydajność wzrostu ($Y_{x/s}$) była wyższa o 10%, a wydajność kwasów ogółem ($Y_{p/s}$) wyższa o 5-12% niż w (B); z kolei właściwe szybkości produkcji kwasów ogółem q_p były wyższe o 10-30% w (B) niż w (A). Chociaż tempo kwaszenia spadało podczas fazy produkcji, to w ostatnim (III) stadium utrzymywało się jeszcze na wysokim poziomie 0.103 h^{-1} i 0.143 h^{-1} dla procesu (A) i (B), odpowiednio. Spadki q_s były wyższe w hodowli (B) niż w (A). Z drugiej strony $Y_{p/s}$ rosła w kolejnych stadiach fazy kwaszenia aż do wysokich wartości 1.12 gg^{-1} w procesie (A) i 0.99 gg^{-1} w procesie (B). Rodzaj użytego inokulum nie miał praktycznie wpływu na stosunek kwasu cytrynowego do izocytrynowego i na właściwą szybkość wzrostu drożdży.