Electronic Journal of Polish Agricultural Universities is the very first Polish scientific journal published exclusively on the Internet, founded on January 1, 1998 by the following agricultural universities and higher schools of agriculture: University of Technology and Agriculture of Bydgoszcz, Agricultural University of Cracow, Agricultural University of Lublin, Agricultural University of Poznan, Higher School of Agriculture and Teacher Training Siedlce, Agricultural University of Szczecin, and Agricultural University of Wroclaw.

ELECTRONIC
JOURNAL
OF POLISH
AGRICULTURAL
UNIVERSITIES

2001
Volume 4
Issue 2
Series
BIOTECHNOLOGY

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ŻAROWSKA B., WOJTATOWICZ M., RYMOWICZ W., ROBAK M. 2001. PRODUCTION OF CITRIC ACID ON SUGAR BEET MOLASSES BY SINGLE AND MIXED CULTURES OF *YARROWIA LIPOLYTICA* **Electronic Journal of Polish Agricultural Universities**, Biotechnology, Volume 4, Issue 2.
Available Online http://www.ejpau.media.pl

PRODUCTION OF CITRIC ACID ON SUGAR BEET MOLASSES BY SINGLE AND MIXED CULTURES OF *YARROWIA LIPOLYTICA*

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ABSTRACT

Three variants of citric acid (CA) biosynthesis on sugar beet molasses by the yeast *Y. lipolytica* were investigated: (I) single cultures of Suc^- strains on inverted molasses, (II) single culture of Suc^+ recombinant strains on non-inverted molasses, (III) mixed cultures of Suc^- and Suc^+ strains on non-inverted molasses.

The aim of the study was to select the most effective production strain and variant of the process.

Two invertase-negative mutant strains of *Y.lipolytica*: A-101-1.22 and A-101-1.31 and two invertase-positive transformants: W29ura3-302 and JM23/pINA169 were used.

The production medium (MM-YE) consisted of 20% (w/v) molasses wort and 1 gL^{-1} of yeast extract (Difco). The cultures were carried out in a Bioflo III bioreactor at working volume 1L, temperature 30°C, pH 5.5, aeration rate 0.5 vvm and agitation rate 500 rpm.

In simple MM-YE medium the best production results were obtained with Suc$^-$ strain A-101-1.22 and with mixed A-101-1.22 + W29ura3-302 culture. In the first process inverted molasses had to be used. The total yield of citric acid production was the highest in these processes and amounted to 0.61 gg^{-1} and 0.55 gg^{-1} , respectively.

Transformant strain W29ura3-302, able to consume sucrose, produced citric acid in simple molasses medium (MM-YE) much slower than other single and mixed cultures of *Y.lipolytica*. The results obtained in much richer production medium (MM-YE, P_P, ura) show potential abilities of the W29ura3-302 strain as a good CA producer. However, further studies are required to optimize the molasses medium for CA synthesis by this strain.

Key words: citric acid, sugar beet molasses, *Yarrowia lipolytica*, mutant strains, invertase-positive transformants, mixed cultures

INTRODUCTION

During the growth on various substrates (glucose, aliphatic hydrocarbons, alcohols, fatty acids, plant oils) at deficiency of nitrogen source, the yeast *Yarrowia lipolytica* accumulates citric acid (CA) together with accompanying isocitric acid (ICA).

In carbohydrate media, the yield of CA biosynthesis by yeast and industrial strains of *Aspergillus niger* is similar.

One of undeniable advantages of using *Y. lipolytica* for CA biosynthesis, instead of traditionally used *Aspergillus niger*, is much higher dynamics of the process [9, 13, 22]. The yeasts are also characterized by a higher, than in case of moulds, tolerance to contamination of culture media with ions of heavy metals and better tolerate high concentration of sugar in the media. The disadvantage is much lower purity of CA fermentation and restricted abilities of *Y. lipolytica* to consume sugars. It restricts the use of such traditional biotechnological raw materials as molasses or whey in the process. There are attempts of eliminating these weak sides of yeast "citric acid fermentation" by the use of mutant strains characterized by lower side production of ICA [2, 11, 20] and of recombinant strains capable to utilize more sugars, e.g. sucrose (Suc$^+$ strains) constructed by molecular techniques [7, 15, 23]. Another possibility is acid or enzymatic hydrolysis of di- or oligosaccharides present in complex media before they undergo the "citric acid fermentation" by yeasts [9, 21]. Mixed culture of yeasts that would complement good biosynthetic properties with an ability to consume, e.g. sucrose can also be used. Numerous examples of the use of mixed yeast cultures may be found in literature concerning production of ethanol from starch [6, 8, 16, 18].

Three variants of CA biosynthesis by *Y. lipolytica* on sugar beet molasses, conducted at a volume of 1 liter, were compared in the present study: (I) single cultures of Suc$^-$ strains on inverted molasses, (II) single culture of Suc$^+$ recombinant strains on non-inverted molasses, (III) mixed cultures of Suc$^-$ and Suc$^+$ strains on non-inverted molasses in order to select the most effective production strain and variant of the process.

MATERIALS AND METHODS

Strains. Two Suc$^-$ strains of *Yarrowia lipolytica* (unable to grow on sucrose as a sole carbon source) were used: A-101-1.22 (reveratant of acetate mutant) and A-101-1.31 (UV – acetate

mutant). Both strains originated from own microorganisms collection of the Department of Biotechnology and Microbiology of the Agricultural University of Wroclaw. Two invertase-positive (Suc^+) transformants of *Y. lipolytica*: W29ura3-302 (MatA, ura 3.302, XPR::SUC2) and JM23/pINA169 (MatB, leu 2-35:XPR::SUC2, lys 5.1, ura 3.8, xpr::LYS5), were kindly provided by Laboratoire de Genetique Moleculaire et Cellulaire, INRA-CNRS, Grignon, France. The latter strain was unable to synthetize CA [23] and was used in the present study as a Suc^- partner of A-101-1.22 strain in mixed culture. The strains were kept on YM agar slants, under paraffin oil, at +4°C.

Media. The growth medium contained in 1 L: 80 g of molasses, 1 g of YE (yeast extract, Difco), 2 g of NH_4Cl, 0.5 g of KH_2PO_4, 1 g of $MgSO_4$ x 7 H_2O. The production medium (MM-YE) consisted of 20% (w/v) water solution (tap water) of sugar beet molasses supplemented with 1 gL^{-1} of YE. Inverted molasses was used for cultivation of Suc^- strains. Sucrose hydrolysis was carried out continuously using a packed-bed bioreactor with immobilized *Saccharomyces cerevisiae* cells [12]. Suc^+ transformant strains and mixed cultures of Suc^+ and Suc^- yeasts were carried out in a medium containing non-inverted molasses. For CA biosynthesis by transformant strain W29ura3-302 a medium MM-YE,P_p,ura enriched with 1.7 gL^{-1} proteose peptone (P_p) and 50 mgL^{-1} uracil (ura) was also used. Concentration of P_p and ura was according to Nicaud et al. [15].

Cultivation techniques. Seed cultures were carried out in 250 mL flasks containing 25 mL of growth medium, on a G-10 shaker (New Brunswick Co, USA) at 160 rev/min, 30°C for 72 h, pH was adjusted to 4 – 5.5 with 2% $CaCO_3$. Two separately grown seed cultures of one or two various yeast strains were used to inoculate 1L of production medium in the bioreactor.

The production cultures were carried out in 3.5L Bioflo III bioreactor (New Brunswick Co, USA) at working volume 1L. The temperature during cultivation was set up at 30^oC, aeration of 0.5 vvm, agitation rate of 500 rev/min and pH maintained at 5.5 by the addition of 30% NaOH. As an antifoam agent Breox (British Petroleum) was used.

Analytical methods. During cultivation in a bioreactor, 5 mL samples were taken every 2 – 12 h for chemical analysis. The biomass [X] was determined using dry weight method at 105°C, citric acid [CA] was determined by pentabromoacetone method [19] and isocitric acid [ICA] was determined by enzymatic method with NADP [5]. Total sugar (after hydrolysis at pH 3 and 80°C for 10 min) and directly reducing sugars were determined using the method of Nelson [14], glucose [Glu] was determined using a diagnostic Glukoza ET kit (POCH, Gliwice). The concentration of sucrose [Suc] was calculated from the differences of concentration of total sugars and directly reducing sugars, whereas fructose concentration [Fru] was calculated from the differences in the concentration of directly reducing sugars and glucose.

RESULTS AND DISCUSSION

Table 1 shows the results of 6 production cultures in basic molasses medium MM-YE with single and mixed cultures of *Y. lipolytica*.

CA biosynthesis in hydrolyzed molasses wort (variant I) was carried out with two invertase-negative strains A-101-1.22 and A-101-1.31. Both mutant strains are characterized by efficient and homogenous CA biosynthesis in synthetic glucose media [20].

Table 1. Final results of citric acid (CA) biosynthesis by single and mixed cultures of *Y. lipolytica* on sugar beet molasses (medium MM-YE).

Parameter	Inverted molasses		Non-inverted molasses			
	Single culture Suc$^-$		Single culture Suc$^+$/mixed culture Suc$^-$ + Suc$^+$			
	A-101-1.22	A-101-1.31	W29ura3-302	A-101-1.22 x W29ura3-302	A-101-1.31 x W29ura3-302	A-101-1.22 x JM23/pINA169
Fermentation time, h	74	94	145	97	113	118
[X], gL^{-1}	13.7	26.3	16.0	18.6	25.3	23.2
[CA], gL^{-1}	58.2	46.9	50.2	62.7	52.1	27.9
[ICA], gL^{-1}	4.5	2.1	2.6	1.0	0.8	0.6
% CA	89.5	92.8	95.1	98.4	98.5	97.9

% CA – % CA in the sum of citric acids (CA + ICA)

In molasses medium MM-YE the strain A-101-1.22 proved to be a better producer of citric acid than the strain A-101-1.31. It produced more CA (58.2 gL^{-1}) at a time of fermentation ca. 24 h shorter (process lasted 74 hours). However, the purity of citric fermentation by these strains on molasses was relatively low; the percentage of ICA in the sum of secreted acids was between 7.2 – 10.5%. In glucose media, the strains did not produce more than 2 – 5% of ICA [22, 17]. Most probably, higher side-production of ICA resulted from the presence of Fe (II) ions which contaminated the molasses and intensified the activity of aconitate hydratase [4].

It was also found that Suc$^-$ strain A-101-1.31 consumed the components of the medium more effectively for production of biomass which was 2 times higher (26.3 gL^{-1}) than in the culture of A-101-1.22 (13.7 gL^{-1}). It resulted in lower final CA accumulation (46.9 gL^{-1}) as after finishing the yeast growth less sugar remained in the medium for CA synthesis.

The process of CA biosynthesis by the invertase-positive W29ura3-302 *Y.lipolytica* transformant was carried out in non-hydrolyzed molasses wort (variant II). The strain was selected in previous studies [23] as a potential production strain for citric fermentation of sucrose media. In the basic MM-YE medium the growth of yeast was limited to the relatively low level, of 16gL^{-1}, which provided excess of carbon source for effective CA production. In these conditions the Suc$^+$ transformant W29ura3-302 accumulated 50.2 gL^{-1} CA and the purity of fermentation was higher (95% CA) than that with invertase-negative strains. However, the dynamics of the process was unsatisfactory as it lasted two times longer (145hours) than the process with A-101-1.22 (Table 1).

The next variant (III) of CA biosynthesis was carried out with mixed culture of Suc$^-$ and Suc$^+$ yeasts on non-hydrolyzed molasses wort (MM-YE). Selection of the strains for mixed culture greatly influenced the effectiveness of CA biosynthesis (Table 1). Among the three combinations of strains, the highest yield of CA was obtained with yeast partners A-101-1.22 and W29ura3-302. The process was relatively short (97 hours), the CA concentration was the highest (62.7 gL^{-1}) at moderate biomass production of 18.6 gL^{-1}. Process of CA biosynthesis by mixed culture of A-101-1.31 + W29ura3-302 strains was longer (113 hours) and characterized by lower CA production (52.1 gL^{-1}). Thus, the strain A-101-1.31 was

a worse partner for the W29ura3-302 transformant than A-101-1.22 due to abundance of cell growth on MM-YE medium, which lowered CA biosynthesis.

The use of Suc^+ transformant JM23/pINA169 deprived of ability to synthesize CA in sucrose media [23], as a partner in mixed culture, did not bring the expected results. During a process which lasted 118 hours, the CA concentration (27.9 gL^{-1}) was not much higher than the biomass concentration (23.2 gL^{-1}). However, it should be noted that all processes with mixed cultures were characterized by high CA selectivity. Citric acid was ca. 98% of the total acids secreted to the medium.

Selected parameters of the kinetics of growth (μ_{max}) and citric acid production (q_{CA} , Q^t_{CA}) and coefficients of CA yield ($Y_{CA/S}$, Y^t_{CA}) are presented in Table 2 and explain the significant differences between the final results of the cultures.

Table 2. Chosen kinetics and yield parameters for the biomass and citric acid production during the cultivation of single and mixed cultures of Y. lipolytica in molasses medium MM-YE

Parameter	Inverted molasses		Non-inverted molasses			
	Single culture Suc^-		Single culture Suc^+/mixed culture Suc^- + Suc^+			
	A-101-1.22	A-101-1.3	W29ura3-302	A-101-1.22 x W29ura3-302	A-101-1.31 x W29ura3-302	A-101-1.22 x JM23/pINA169
μ_{max}, h^{-1}	0.21	0.35	0.23	0.46	0.38	0.47
q_{CA}^{Glu}, $gg^{-1}h^{-1}$	0.120	0.110	0.059	0.100	0.063	0.026
q_{CA}^{Fru}, $gg^{-1}h^{-1}$	0.067	0.050	0.014	0.030	0.015	0.010
Q^t_{CA}, $gL^{-1}h^{-1}$	0.78	0.50	0.37	0.64	0.46	0.24
$Y_{CA/S}$, gg^{-1}	0.89	0.92	0.61	0.87	0.92	0.58
Y^t_{CA}, gg^{-1}	0.61	0.44	0.42	0.55	0.45	0.26

μ_{max} – specific growth rate during exponential phase (h^{-1}); q^{Glu}_{CA} – specific rate of citric acid production during glucose consumption ($gg^{-1}h^{-1}$); q^{Fru}_{CA} – specific rate of citric acid production during fructose consumption ($gg^{-1}h^{-1}$); Q^t_{CA} – total productivity of citric acid biosynthesis ($gL^{-1}h^{-1}$); Y^t_{CA} – total citric acid yield coefficient (g CA produced / g total sugar consumed); $Y_{CA/S}$ – citric acid yield coefficient (g produced CA / g sugar consumed for acid production only, assuming that $Y_{X/S}$ = 0.45 gg^{-1}).

From among the single cultures used in the investigation, mutant strain A-101-1.31 grew in the most dynamic way in the MM-YE medium (μ_{max} = 0.35 h^{-1}) (Table 2). Higher growth rates, μ_{max} = 0.38 – 0.47 h^{-1}, were observed in each of the mixed cultures tested. It proved better complementation of strain features and better adaptation of mixed cultures to production medium. High growth rate did not always correlate with high CA production rate.

In all processes, two phases of constant CA production were observed: the first one fell on glucose consumption period and the second one, during fructose consumption. The specific rate of CA production during glucose consumption (q^{Glu}_{CA}) was highest in processes with invertase-negative strains A-101-1.22, A-101-1.31 and in mixed culture of A-101-1.22 with Suc^+ W29ura3-302 transformant and amounted to 0.12 $gg^{-1}h^{-1}$, 0.11 $gg^{-1}h^{-1}$ and 0.10 $gg^{-1}h^{-1}$, respectively. Such values of specific rate of CA production in synthetic glucose media were found only in the best production strains of *Y.lipolytica* [3, 10, 13]. All the above processes were also characterized by the highest specific rate of CA production during fructose

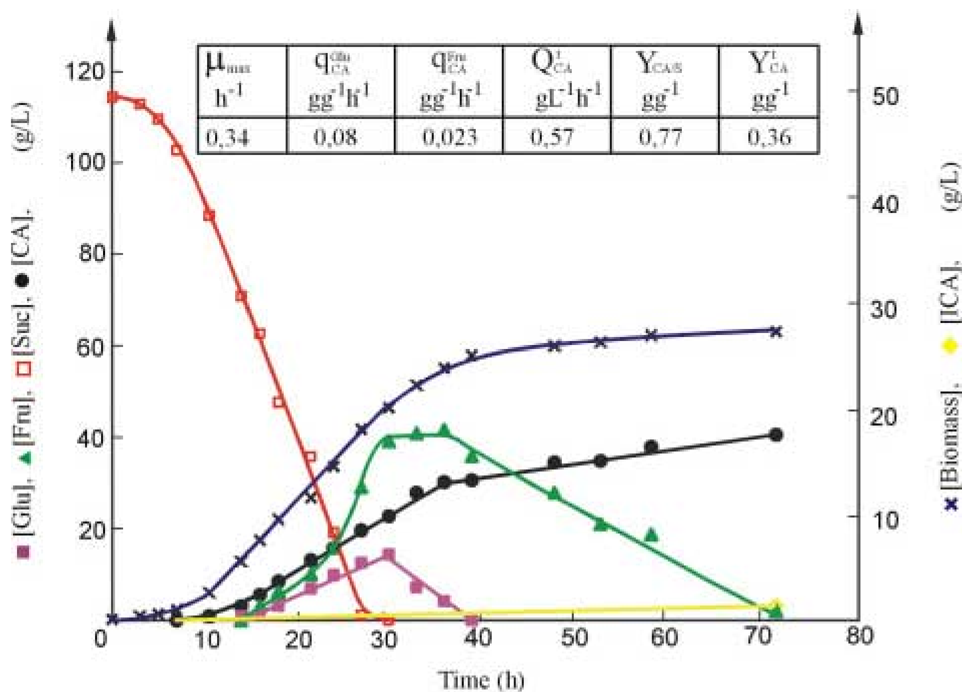
consumption, q^{Fru}_{CA} ranged from 0.03 $gg^{-1}h^{-1}$ to 0.067 $gg^{-1}h^{-1}$ and high efficiency of sugar conversion to CA ($Y_{CA/S} \approx 0.9$ gg^{-1}).

It seems that good production results obtained in analyzed fermentation processes resulted from moderate yeast growth (Table 1), as sugar consumption for biomass production was lower and there was more sugar for CA formation. Hence, both total citric acid yield coefficient (Y^{t}_{CA}) and total productivity (Q^{t}_{CA}) were highest in the processes of CA biosynthesis by single culture A-101-1.22 on inverted molasses and by mixed culture A-101-1.22 and W29ura3-302 on non-inverted molasses.

From among the three variants of CA biosynthesis by *Y. lipolytica* on sugar beet molasses, the best solution would be to use a strain that is both an efficient CA producer and sucrose consumer. However, the Suc^{+} transformant W29ura3-302 produced CA in basic molasses medium MM-YE much slower than other single and mixed cultures of *Y. lipolytica* in which it was a partner strain. It was supposed that it might have resulted from deficiency of proteins and peptides, indispensable for efficient expression of invertase gene in this strain [15] and of growth factors, uracil rather than tiamine , as the latter was provided at sufficient amount with 1 gL^{-1} YE [21].

As a result, we decided to carry out an extra cultivation in a medium supplemented with 1.7 gL^{-1} proteose peptone (P_p) and 50 mgL^{-1} uracil (ura) (Fig. 1).

Fig. 1. Production of biomass [X], citric acid [CA] and isocitric acid [ICA], sucrose degradation [Suc] and consumption of glucose [Glu] and fructose [Fru] during cultivation of Suc^{+} transformant strain of *Y. lipolytica* W29ura3-302 in molasses medium MM-YE,P_p,ura.
Kinetics and yield parameters as in Table 2.



μ_{max} h^{-1}	q^{Glu}_{CA} $gg^{-1}h^{-1}$	q^{Fru}_{CA} $gg^{-1}h^{-1}$	Q^{t}_{CA} $gL^{-1}h^{-1}$	$Y_{CA/S}$ gg^{-1}	Y^{t}_{CA} gg^{-1}
0,34	0,08	0,023	0,57	0,77	0,36

The process of CA production by W29ura3-302 transformant in enriched medium MM-YE,P_p,ura was significantly shorter than that in basic MM-YE medium and lasted only 72 hours. From the beginning of cultivation the transformant strain dynamically hydrolyzed sucrose and in the 27th hour no sucrose was present in the medium. The profiles of cell

growth and CA production on sucrose monomers were similar to these previously found for other strains of *Y. lipolytica* growing in molasses and sucrose media [this work, 9, 23]. The transformant strain first consumed glucose for cell growth and CA synthesis, then fructose, but only for citrate production. Specific growth rate and specific CA production rate were significantly higher in MM-YE,P_p,ura than in MM-YE medium. Such modification of the medium proved to be unfavorable as it resulted in excessive yeast growth. The biomass in this culture reached a level as high as 27 gL^{-1}. It had a negative effect on total citric acid yield (Y^t_{CA} = 0.36 gg^{-1}) and on its final concentration, which decreased from 50.2 gL^{-1} (MM-YE) to 40.9gL^{-1}.

The results obtained in the study show a need of further research on optimizing molasses medium for CA biosynthesis by Suc^+ transformant of *Y. lipolytica* W29ura3-302.

CONCLUSIONS

1. Citric acid biosynthesis by *Y. lipolytica* A-101-1.22 in 20% (w/v) molasses wort supplemented with 0.1% of yeast extract was characterized by the highest productivity (0.78 $gL^{-1}h^{-1}$) and total citric acid yield (0.61 gg^{-1}), but inverted molasses had to be used.
2. Mixed culture of A-101-1.22 strain and invertase-positive transformant W29ura3-302 made it possible to use non-inverted molasses for CA production. The productivity (0.64 $gL^{-1}h^{-1}$), total CA yield (0.55 gg^{-1}) and purity of the bioprocess (98.4% CA) were significantly higher than in the process with single culture of W29ura3-302.
3. The invertase-positive transformant W29ura3-302 may potentially be used for CA production on sugar beet molasses but further research is required to intensify the process.

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