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CITRIC ACID BIOSYNTHESIS BY *YARROWIA LIPOLYTICA* A-101-1.31 UNDER DEFICIENCY OF VARIOUS MEDIUM MACROCOMPONENTS

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ABSTRACT

The objective of the study was to examine the potential of *Yarrowia lipolytica* A-101-1.31 growth control via the concentration of the nitrogen, phosphorus or sulphur source, as well as to assess the efficiency of each source separately and under simultaneous deficiency of nitrogen and phosphorus.

The experimental study has produced the following findings: nitrogen, phosphorus and sulphur enabled the control of *Y.lipolytica* A-101-1.31 growth. Citric acid biosynthesis occurred irrespective of the growth-limiting factor used. The yeast cells produced citric acid with the desired efficiency only under conditions of nitrogen (q_{CA}^{g}=0.071 $gg^{-1}h^{-1}$) or sulphur (Y_{CA}^{P}=0.74 gg^{-1}) deficiency. The smallest changes in the elemental composition were observed when the yeast grew under nitrogen limitation. Elemental composition variations were the most distinct when the yeast biomass was cultivated under phosphorus deficiency. A low deficiency of phosphorus in the nitrogen deficient had a stimulating effect on the acid-producing of the growing cells (q_{CA}^{g}=0.089 $gg^{-1}h^{-1}$).

Key words: citric acid, *Yarrowia lipolytica*, growth limitation.

INTRODUCTION

The ease with which yeasts (and this includes the strain *Yarrowia lipolytica*) produce citric acid in an environment where carbon and energy sources occur in excess while cellular growth experience in the literature. When use is made of synthetic media for the biosynthesis of citric acid, it is a common practice to control yeast growth by limiting the availability of the inorganic nitrogen source [1]. According to some investigators [11, 18], biomass growth can also be controlled by the macrocomponents of the medium that act as phosphorus, sulphur or magnesium sources. It has also been found that the type of the growth limiting factor has a noticeable effect not only on the capability of the yeasts to produce citric acid but also on the extent of conversion of the carbon and energy source into citric acid by the yeasts used [11]. Many authors [3, 4, 5, 8, 12, 13] have investigated fermentative production of citric acid by yeasts, using media not only with nitrogen but also phosphorus limitation. However, some of our previous studies on continuous citric acid biosynthesis carried out under similar conditions [15, 16, 19] have

revealed that the stability of these processes is poor. This finding implies that the choice of the growth-limiting factor is of prime importance to the design of continuous cultivation systems, and so is the consideration of the widest possible spectrum of yeast growth limiting factors when designing processes of citric acid biosynthesis.

The aim of the study reported on in this paper was to examine the possibility of *Y.lipolytica* A-101-1.31 growth control by various macrocomponents of the medium used (concentration of nitrogen, phosphorus or sulphur source alone; concentration of both nitrogen and phosphorus sources). Another major objective was to assess the efficiency of citric acid production from a glucose medium by this yeast strain under limitation of the chemical elements mentioned.

MATERIALS AND METHODS

Microorganism. The yeast strain *Yarrowia lipolytica* A-101-1.31 used in this study comes from the collection of the Department of Biotechnology and Food Microbiology, Agricultural University of Wroclaw, Poland. It is a mutant (oct$^-$) unable to grow on acetate as the sole carbon and energy source, and has been obtained from the strain A-101 by exposure to UV radiation. Compared to its parent, the strain is characterized by a notably enhanced purity of citric fermentation on a glucose medium. The yeast strain was maintained on YM slants with paraffin at 4°C.

Media. The composition of the culture media applied is shown in Table 1. Each medium was sterilized at 121°C for 20 minutes.

Table 1. Culture media used

Medium name and composition	Application
YM agar: yeast extract, 3g; malt extract, 3g; peptone, 5g; glucose, 20g,; agar, 20g; distilled water,1L	yeast maintenance
Growth medium: glucose,100g; NH_4Cl, 8.6 → 0.2g; KH_2PO_4,3 → 0.025g; $MgSO_4$ x $7H_2O$,1 → 0.025g; $MgCl_2$ x $6H_2O$, 0.163 → 0.82g; KCl, 1.64 → 0g; yeast extract, 1g; thiamine-HCl (B_1), 200µg; $FeSO_4$ x $7H_2O$, 2mg; $ZnSO_4$ x $7H_2O$, 2.5mg; $MnSO_4$ x $1H_2O$, 1mg,; $CuSO_4$ x $5H_2O$, 0.1mg; distilled water, 1L; $CaCO_3$, 0.5g/25mL	growth cultures: detrmination of the relationship between biomass yield and N, P, S source concentration
Inoculum medium: glucose, 40g; NH_4Cl, 3g; KH_2PO_4, 0.5g; $MgSO_4$ x $7H_2O$.1g; yeast extract, 1g; $FeSO_4$ x $7H_2O$, 2mg; $ZnSO_4$ x $7H_2O$, 2.5mg; $MnSO_4$ x $1H_2O$, 1mg; $CuSO_4$ x $5H_2O$, 0.1mg; distilled water, 1L	seed cultures
N deficient medium: glucose, 100g; NH_4Cl, 1.5g; KH_2PO_4, 3 g; $MgSO_4$ x $7H_2O$, 1g; yeast extract, 1g; thiamine-HCl (B_1), 200µg; $FeSO_4$ x $7H_2O$, 2mg; $ZnSO_4$ x $7H_2O$, 2.5mg; $MnSO_4$ x $1H_2O$, 1mg; $CuSO_4$ x $5H_2O$, 0.1mg; distilled water, 1L	citric acid biosynthesis
S deficient medium: glucose, 100g; NH_4Cl, 8.6g; KH_2PO_4, 3 g; $MgSO_4$ x $7H_2O$, 0.1g; $MgCl_2$ x $6H_2O$, 0.163; yeast extract, 1g; thiamine-HCl (B_1), 200µg; $FeSO_4$ x $7H_2O$, 2mg; $ZnSO_4$ x $7H_2O$, 2.5mg; $MnSO_4$ x $1H_2O$, 1mg; $CuSO_4$ x $5H_2O$, 0.1mg; distilled water, 1L	
P deficient medium: glucose, 100g; NH_4Cl, 8.6g; KH_2PO_4, 0.025g; $MgSO_4$ x $7H_2O$, 1g; KCl, 1.64; yeast extract, 1g; thiamine-HCl (B_1), 200µg; $FeSO_4$ x $7H_2O$, 2mg; $ZnSO_4$ x $7H_2O$, 2.5mg; $MnSO_4$ x $1H_2O$, 1mg; $CuSO_4$ x $5H_2O$, 0.1mg; distilled water, 1L	
NP deficient medium: glucose, 100g; NH_4Cl, 1.5g; KH_2PO_4, 0.2g; $MgSO_4$ x $7H_2O$, 1g; yeast extract, 1g; thiamine-HCl (B_1), 200µg; $FeSO_4$ x $7H_2O$, 2mg; $ZnSO_4$ x $7H_2O$, 2.5mg; $MnSO_4$ x $1H_2O$, 1mg; $CuSO_4$ x $5H_2O$, 0.1mg; distilled water, 1L	

Cultivation methods

Growth cultures. To establish the relation between biomass yield and concentration of the N, P, S source in the growth medium, yeasts were cultured in 250 mL Erlenmeyer flasks (each containing a 25 mL portion of the medium) on an Elpan rotational shaker at 160 rev min^{-1} and 30°C for 7 days. The inocula for the production cultures were prepared on the shaker under similar conditions. Inoculum cultures were carried out for 48 h. The biomass of two shake flask cultures was inoculated onto 1 L of the production medium in the bioreactor.

Production cultures. Citric acid biosynthesis was performed in a 3.5 L volume BIOFLO III bioreactor (New Brunswick), with a 1 L volume of the nutrient medium, at 30°C and a pH set at 5.5 (controled automatically with 10 M NaOH), at a stirrer speed of 500 rev min^{-1} and an aeration rate of 0.2 vvm.

Determination of biomass. In growth cultures and production cultures biomass was determined gravimetrically after drying in a drier at 105°C.

Determination of citric acid. Citric acid was determined by the pentabromoacetone method [17].

Determination of isocitric acid. Isocitric acid was determined by an enzymatic method, in the presence of isocitrate dehydrogenase [7].

Determination of glucose. Glucose was determined by enzymatic analysis, with a ready-to-use diagnostic unit (Glucoza et New, POCh Gliwice).

Determinations of carbon, nitrogen, hydrogen, sulphur and phosphorus in the biomass. The content of chemical elements in the biomass was analyzed at the end of each cultivation. C, H, N, S were determined by gas chromatography, using a CHNS EA-1110 analyzer (CE Instruments). Phosphorus was analyzed with an ICP-AES Liberty 220 spectrophotometer (Varian). Minerlization of the biomass samples was determined at increased pressure in a CEM-MDS 2000 microwave mineralizer.

List of symbols used

μ_{max} = maximal spcific growth rate (h^{-1})
$Y_{X/S}$ = yield of cell growth (gg^{-1})
q_{CA}^{g} = specific rate of acid production in the yeast growth phase ($gg^{-1}h^{-1}$)
q_{CA}^{n-g} = specific rate of acid production after termination of yeast growth ($gg^{-1}h^{-1}$)
Y_{CA}^{P} = yield of citric acid (gg^{-1})
Y_{CA}^{t} = total yield of the process (gg^{-1})

RESULTS AND DISCUSSION

The results of this study have substantiated the potentiality for *Y.lipolytica* A-101-1.31 growth control with nitrogen, phosphorus and sulphur (Fig. 1). Since KH_2PO_4 was used as the source of phosphorus, the deficiency of potassium in the medium was made up by the addition of KCl; the deficiency of magnesium in the sulphur deficient medium (where sulphur sourced from $MgSO_4$ x $7H_2O$) was compensated by adding $MgCl_2$ x H_2O). The plots relating the biomass yield to the concentrations of particular chemical substances used are of a linear nature. Thus the biomass yield was influenced primarily by the changes in the amount of nitrogen, and was found to vary from 2.5 to 26.2 gL^{-1} over the investigated range of nitrogen concentrations. However, any change in the concentration of the phosphorus source in the medium had a very poor effect on the control of the biomass yield. Even with an extremely low phosphorus dose (0.025gL^{-1}) KH_2PO_4), biomass yield was still very high, amounting to 14 gL^{-1}.

The relations established in the study helped to design such processes of citric acid biosynthesis where the maximal biomass yield approached 15 gL^{-1}, irrespective of the limiting substance used.

Four batch processes of citric acid biosynthesis were examined: (A) with a nitrogen deficient medium (1.5 gL^{-1} NH_4Cl = 0.3925 gL^{-1} N); (B) with a phosphorus deficient medium (0.025 gL^{-1} KH_2PO_4 = 0.0057 gL^{-1} P); (C) with a sulphur deficient medium (0.1 gL^{-1} $MgSO_4x7H_2O$ = 0.013 gL^{-1} S) and for the purpose of comparison (D) with a medium deficient in both nitrogen and phosphorus (1.5 gL^{-1} NH_4Cl and 0.2 gL^{-1} KH_2PO_4) (Fig. 2).

Fig. 1. Effect of nitrogen, phosphorus and sulphur concentrations on the growth of *Y. lipolytica* A-101-1.31 cells

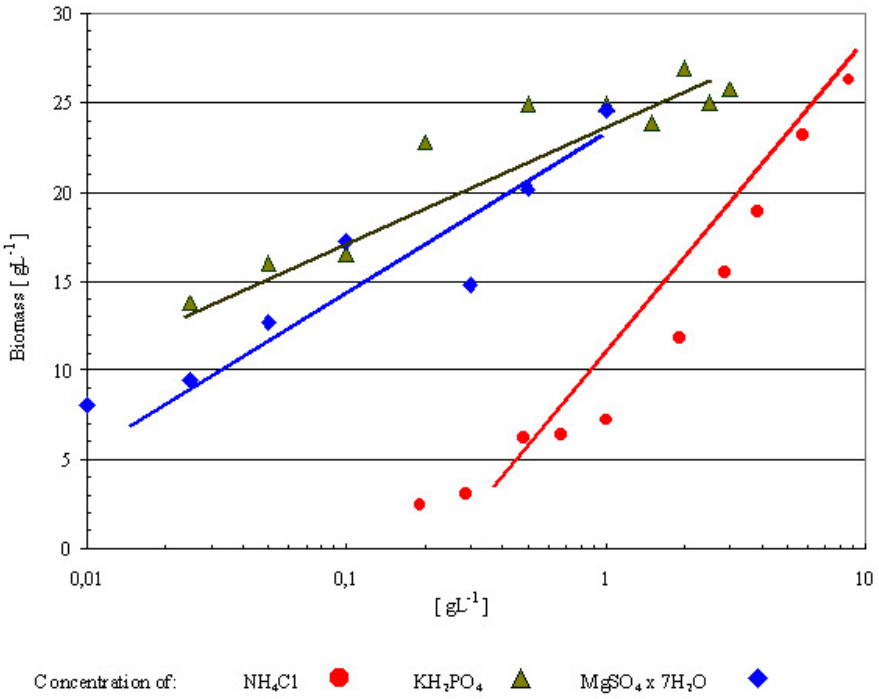
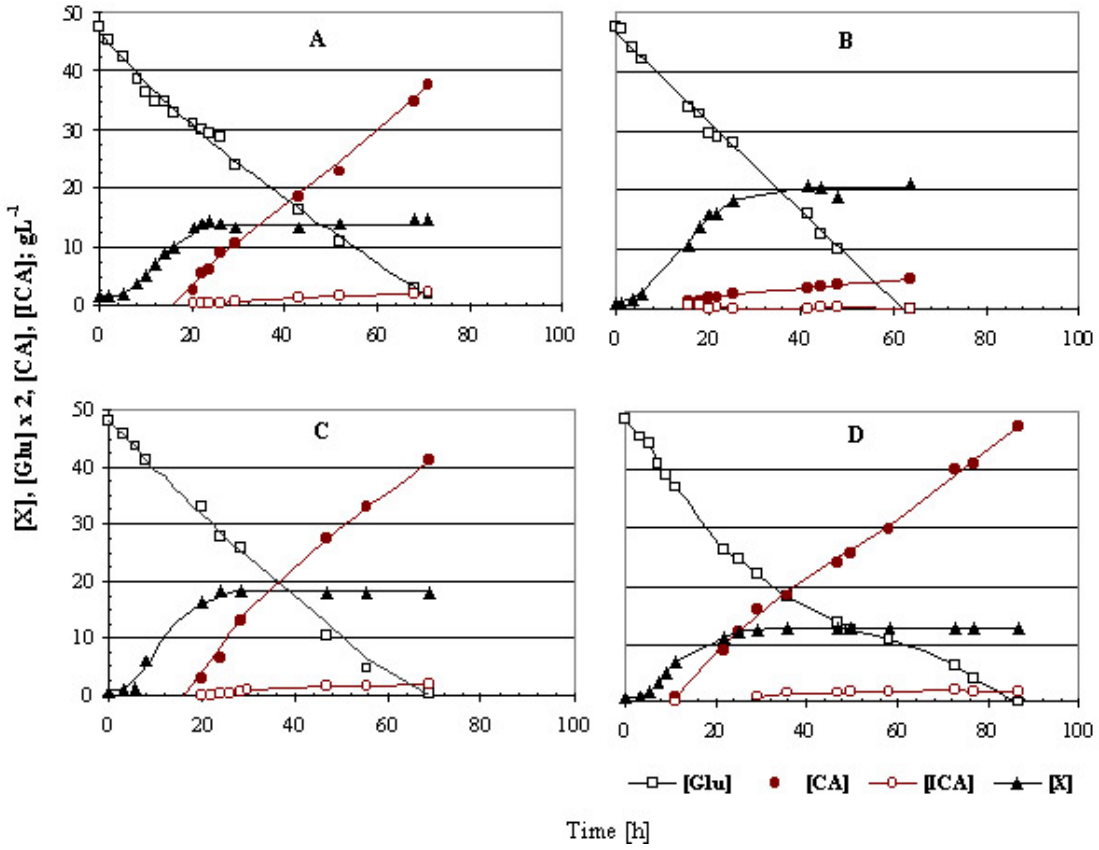


Fig. 2. Production of biomass [X], citric acid [CA] and isocitric acid [ICA] and degradation of glucose [Glu] in a batch culture of *Y. lipolytica* A-101-1.31 at deficient: nitrogen (A), phosphorus (B), sulphur (C), nitrogen and phosphorus (D)



The results of fermentation show that citric acid biosynthesis occurred regardless of which growth limiting substance was used, but the synthesis of this metabolite was the most efficient under conditions of nitrogen or sulphur deficiency. When yeast growth was controlled by the nitrogen source, the amount of citric and isocitric acid produced during 71 h totalled 37.5 gL^{-1} and 2 gL^{-1}, respectively (Fig. 2A). When the process was carried out using a sulphur deficient medium, complete degradation of glucose was observed after 69 h. Over that period, the production of citric and isocitric acid amounted to 41.2 gL^{-1} and 1.8 gL^{-1}, respectively (Fig. 2C). Under phosphorus limitation, the process terminated after 63.5 h with a citric acid production of 4.8 gL^{-1} only (Fig. 2B). For comparison, when biomass was grown on the medium deficient in both nitrogen and phosphorus, the process had the longest duration and produced the greatest amount of citric acid, 47.5 gL^{-1} (Fig. 2D).

Y.lipolytica A-101-1.31 was found to produce citric acid with a specific rate which was higher in the growth phase (q_{CA}^{g}) than in the stationary phase (q_{CA}^{n-g}). This finding has been reported in the literature [4, 18, 20] and is of importance in the case of a single-stage continuous culture where citric acid is produced by growing yeast cells only.

In the study reported on in this paper, under conditions of yeast cultivation on a medium deficient in one macrocomponent, the highest productivity was attained both in the growth phase (q_{CA}^{g} = 0.071 gg^{-1}) and in the stationary phase (q_{CA}^{n-g} = 0.048 $gg^{-1}h^{-1}$) when the medium used was deficient in nitrogen. A low phosphorus deficiency in the medium used for a comparative study, where yeast growth was limited by the nitrogen source, stimulated the productivity of citric acid biosynthesis by growing cells (Table 2). There have also been reports demonstrating that a slight deficiency of phosphorus in nitrogen-limited *Y.lipolytica* A-101 cultures growing on a glucose or n-hexadecane medium noticeably enhances productivity [14, 18]. A similar effect has been obtained with molasses media where the compensation of the natural phosphorus deficiency by the addition of KH_2PO_4 brought about a decrease in the rate and yield of citric acid biosynthesis by a variety of *Y. lipolytica* strains [21]. Another research reported in the literature [6] was revealed a very high total citric acid yield when use was made of a phosphorus deficient hydrocarbon medium with excess of NH_4^+ ions.

Table 2. Major kinetic and yield parameters for biomass and citric acid production during batch cultivation of *Y. lipolytica* A-101-1.31 related to growth conditions

Parameter	Medium			
	N deficient medium	P deficient medium	S deficient medium	NP deficient medium
μ_{max}, h^{-1}	0.15	0.2	0.23	0.23
$Y_{X/S}$, gg^{-1}	0.375	0.4	0.45	0.4
q_{CA}^{g}, gg^{-1} h^{-1}	0.071	0.02	0.067	0.089
q_{CA}^{n-g}, gg^{-1} h^{-1}	0.048	0.005	0.038	0.046
Y_{CA}^{P}, gg^{-1}	0.69	0.11	0.74	0.71
Y_{CA}^{t}, gg^{-1}	0.4	0.05	0.43	0.47

In our study, not only cellular growth yield ($Y_{X/S}$) but also citric acid yield in the acid production phase (Y_{CA}^{P}) were the highest under sulphur limitation, amounting to 0.45 gg^{-1} and 0.75 gg^{-1}, respectively. Our results are in agreement with those published by McKay et al. [11]. In their experiments with a culture of the strain Y.lipolytica IMK 2, citric acid yield obtained on a nitrogen or sulphur deficient medium was twice as high as the one achieved using a phosphorus deficient medium.

In general, the values of the kinetic parameters, as well as those of the efficiency of citric acid production by the strain examined in our experimental study, fell in the range established in the literature for other strains. The specific rate of citric acid synthesis from glucose media deficient in nitrogen or nitrogen and phosphorus varied over a wide range: from 0.028 to 0.07 $gg^{-1}h^{-1}$, the values of Y_{CA}^{P} ranging between 0.2 and 0.7 gg^{-1} [2, 5, 9, 10]. Notably higher values of these parameters, q_{CA}=0.12 $gg^{-1}h^{-1}$ and Y_{CA}^{P}=0.78 gg^{-1}[19], have been reported only for the strain *Y.lipolytica* A-101.

Our examinations of the elemental composition of the biomass have revealed that particular macrocomponents of the medium occurred at limiting concentrations. Upon comparing the composition of the biomass from particular cultivations with that of the yeasts cultured in a growth medium of a balanced elemental composition (C:N:P:S≈10:1:0.2:0.1), it became obvious that the least distinct changes were those in the elemental composition of the yeasts grown on the nitrogen deficient medium; the biomass showed a reduced level (3.07%) of this element alone (Table 3).

Table 3. Elemental composition of *Y.lipolytica* A-101-1.31 biomass accumulated during batch biosynthesis of citric acid related to growth conditions in production medium and full medium

Medium	Concentration (% ss)				
	C	**H**	**N**	**P**	**S**
N deficient medium	48.09	7.32	3.07	2.09	0.38
P deficient medium	56.05	7.29	4.97	0.16	0.46
S deficient medium	44.65	6.87	5.76	1.91	0.06
PN deficient medium	48.5	7.75	3.42	0.31	0.22
Full medium (C : N : P : S ≈ 10 : 1 : 0.2 : 0.1)	47.14	6.90	7.28	2.08	0.25

As already mentioned, under conditions of phosphorus deficiency the activity of citric acid production biosynthesis was very poor. The examination of the biomass elemental composition implies that the rearrangement of the cell structures may have been the largest under such conditions. The biomass showed a 12-fold lower phosphorus content, as well as a reduced nitrogen level, as compared to the biomass produced from a medium of a balanced elemental composition.

CONCLUSION

The cultivation of the *Y.lipolytica* A-101-1.31 strain on a synthetic glucose medium for the biosynthesis of citric acid can be controlled by the concentration of the nitrogen, phosphorus or sulphur source. The quantity of NH_4Cl, KH_2PO_4 and $MgSO_4x7H_2O$ applied amounted to 1.5 to 2.0 gL^{-1}, 0.025 to0.05 gL^{-1} and 0.1 gL^{-1}, respectively.

The yeasts produced citric acid with a high efficiency only from a nitrogen or sulphur deficient medium.

A slight deficiency of phosphorus (0.2 gL^{-1} KH_2PO_4) in the nitrogen deficient medium (1.5 gL^{-1} NH_4Cl) had a favourable effect on the production of citric acid by the growing cells; the acid-producing activity of the cells rose from 0.071 $gg^{-1}h^{-1}$(measured in the process with a nitrogen deficient medium) to 0.089 $gg^{-1}h^{-1}$ (determined using a nitrogen deficient medium with a slight deficiency in phosphorus).

Yeasts growing on a nitrogen deficient medium showed the smallest changes in their elemental composition: there was only a reduction in the nitrogen level of the biomass. The greatest changes were observed in the composition of the biomass grown on a phosphorus deficient medium there was a reduction not only in nitrogen but also in phosphorus.

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