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conference paper

Metabolic Engineering of the Glycolytic Pathway in *Aspergillus niger*

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Summary

Citric acid is produced by fermentation employing the filamentous fungus Aspergillus niger. The biosynthetic pathway involved in conversion of sugar substrates to citric acid includes glycolysis and part of the tricarboxylic acid cycle. This paper describes some of the recent developments for improvement of A. niger strains by genetic engineering. Two aspects are discussed: characterisation of A. niger primary metabolism, and overexpression of glycolytic genes in A. niger with subsequent analysis of citric acid production by the modified strains.

Keywords: metabolic engineering, citric acid, *Aspergillus niger*, glycolysis

Introduction

Industrial-scale production of citric acid is performed mainly by the fermentation processes involving the filamentous fungus *Aspergillus niger (1-3)*. Although production yields are generally high, the process may still be improved by increasing the production rate, *i.e.* by decreasing fermentation time. Rate improvements may be realised by process engineering or by strain improvement programmes. In this paper we will discuss several approaches to strain improvement with emphasis on metabolic engineering. Traditional strain improvement programmes mostly involve mutagenesis and selection. These classical genetic methods have provided significant improvements, both in yield and in rate, resulting in very efficient citric acid producing strains. Another approach is genetic engineering of fungal metabolism involved in citric acid biosynthesis. In contrast to random mutagenesis, metabolic engineering is a more rational approach, as particular metabolic steps are modified to increase the metabolic flux through the pathway leading to citric acid formation. Two ways of speeding up citric acid biosynthesis are investigated: *(i)* increasing the flux through the central pathway, *e.g.* by overproduction of the enzymes involved, and *(ii)* decreasing the fluxes through branches of the main pathway resulting in decreased formation of by-products.

Decreased by-product formation

Conversion of substrate into other compounds than citric acid is unwanted because by-product formation may reduce the rate and final yield, and because these compounds may complicate recovery of citric acid from the broth. Two groups of compounds are readily overproduced by *A. niger*, namely organic acids and polyols. During citric acid fermentation formation of gluconic acid and oxalic acid can be problematic. Gluconic acid is formed from glucose by glucose oxidase. Glucose oxidase is localised in the extracellular matrix (Fig. 1) explaining the fast conversion of glucose to gluconic acid by *A. niger (4)*. A few years ago a glucose oxidase mutation, *gox*C17 was isolated in our laboratory employing a colony screening method *(5)*. Strains with this mutation lack an active glucose oxidase and do not produce gluconic acid from glucose. This mutation eliminates gluconic acid as a by-product during citric acid fermentations using substrates containing glucose. Oxalic acid is produced by oxaloacetate hydrolase, which is a cytoplasmic enzyme *(6)*. Biosynthesis of oxaloacetate hydrolase is regulated by external pH. Induction of the enzyme is optimal at pH 5 to 6, whereas a very low oxaloacetate hydrolase activity is observed at pH = 2 *(6)*. An oxalate non-producing strain would be advantageous, particu-

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larly in molasses-based fermentations in which the initial pH is 5 to 6.

Polyols, such as glycerol, erythritol and mannitol, are usually not produced in very large amounts and, moreover, are reconsumed upon exhaustion of the sugar substrate *(7-9)*. Thus, polyol compounds are not a major problem for the final yield of citric acid as long as the sugar substrate is completely consumed. The subsequent production and reconsumption of polyols may, however, reduce the rate of citric acid production. Polyols probably have several important functions in fungal physiology. There are indications that they function in osmoregulation and as reserve carbohydrates *(7)*. It might, therefore, be difficult to block polyol formation without seriously disturbing cellular physiology. Nevertheless, we have recently isolated a gene encoding mannitol-1-phosphate dehydrogenase from *A. niger* (*mpd*). Mannitol-1-phosphate dehydrogenase catalyses conversion of the glycolytic intermediate fructose-6-phosphate to mannitol-1-phosphate and is the first step in mannitol biosynthesis (Fig. 1). We intend to construct an *mpd* mutant by disruption of the gene. In such a strain mannitol formation would be blocked.

Engineering of glycolysis

The metabolic pathway involved in biosynthesis of citric acid from hexoses in *A. niger* is well established *(2,3)*. Following uptake, hexoses are degraded mainly *via* glycolysis yielding pyruvate (Fig. 1). Part of the pyruvate is then converted to acetyl-CoA, part to oxaloacetate. Finally, these two compounds are condensed to citric acid, which is, under a particular set of conditions, secreted and accumulated in the medium.

A rational strategy for improvement of the process requires knowledge of which steps to modify. In the case of citric acid biosynthesis by *A. niger*, quite a few enzymes have been studied. These data as such do not predict which steps may be bottlenecks but should be used in a quantitative analysis of the whole pathway *(10–12)*. Construction of a mathematical model of the metabolism involved will enable determination of the flux control distribution over the enzymes in the pathway and could provide a guideline for genetic engineering of the metabolism, *e.g.* overexpression of certain enzymes, that would lead to improved production. Recently, a few attempts have been made to analyse flux control in citric acid biosynthesis by *A. niger (13–16)*. Torres performed modelling of the first part of the pathway, *i.e.* up to pyruvate and concluded from flux optimisation calculations that simultaneous overproduction of seven enzymes was required for a significant increase in flux *(16)*. At present this is technically still complicated.

Although the pathway is known, a number of enzymes involved have not been properly characterised. Such a lack of data hinders an accurate analysis of the metabolism. Therefore, we are purifying and characteri-

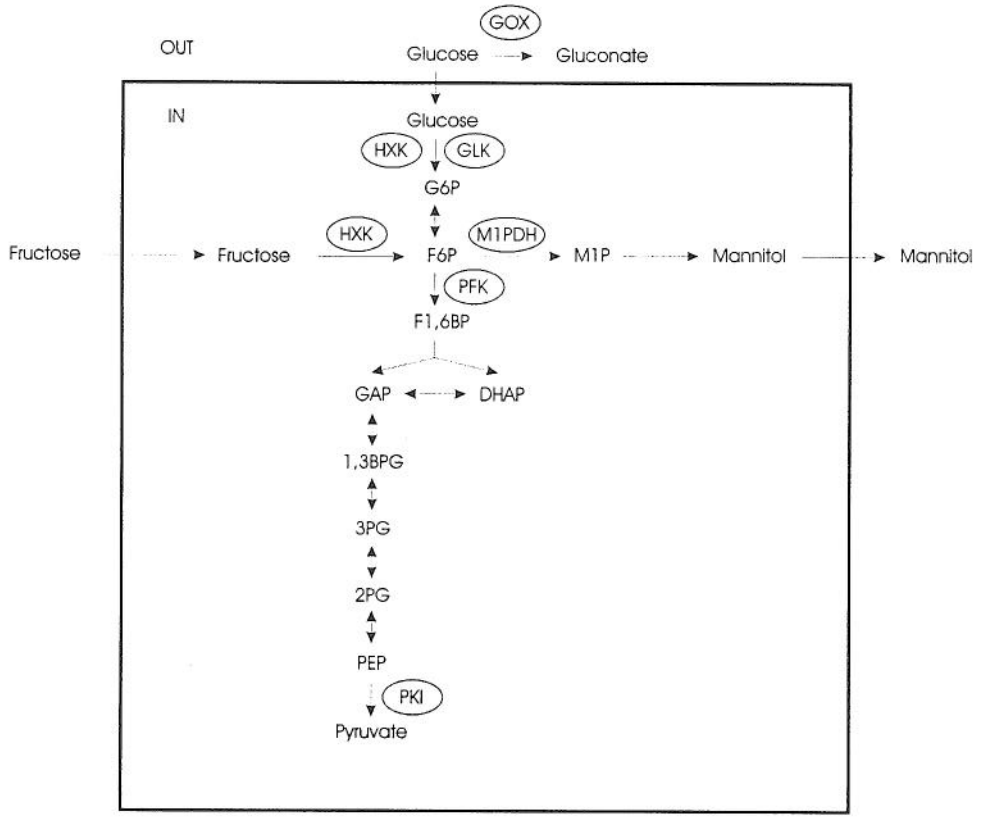

Fig. 1. Schematic representation of glycolysis and mannitol biosynthesis in *A. niger*. The following enzymes are depicted: GOX, glucose oxidase; HXK, hexokinase; GLK, glucokinase; M1PDH, mannitol-1-phosphate dehydrogenase; PFK, phosphofructokinase and PKI, pyruvate kinase.

sing glycolytic enzymes, which will provide more data for a reliable metabolic model. For the actual genetic engineering of glycolysis we have isolated a number of genes encoding glycolytic enzymes: *hxk*A (hexokinase), *glk*A (glucokinase), *pfk*A (phosphofructokinase), *gpd*A (glyceraldehyde-3-phosphate dehydrogenase), *pgk*A (phosphoglycerate kinase) and *pki*A (pyruvate kinase).

Hexose phosphorylation in *A. niger*

A. niger phosphofructokinase and pyruvate kinase have been characterised quite well, but this is not the case for the hexose phosphorylating enzymes. Until recently, all data indicated the presence of a single hexose phosphorylating enzyme in *A. niger (17)*. However, we have now found that *A. niger* possesses a hexokinase and a glucokinase. Several observations indicate the presence of two separate enzymes. First, we isolated the *A. niger* glucokinase gene (*glk*A) by a PCR strategy and found that multicopy *glk*A transformants did have a high glucose phosphorylating activity, but fructose phosphorylation was hardly higher than observed in a wild-type strain *(18)*. Glucokinase was purified from the multicopy transformant by dye-affinity chromatography and appeared to have a high affinity for glucose (K_m = 0.063 mM at pH = 7.5) and a very low affinity for fructose (K_m = 120 mM at pH = 7.5), rendering *in vivo* fructose phosphorylation by glucokinase negligible. In addition, the enzyme was not inhibited by trehalose-6-phosphate, unlike many hexokinases *(19)*.

Subsequently, the *A. niger* hexokinase gene was isolated using heterologous hybridisation with an *A. nidulans hxk*A probe. The *A. nidulans hxk*A gene was isolated by complementation of an *A. nidulans* mutant lacking hexokinase activity *(20)*. An *A. niger hxk*A multicopy transformant has high glucose- and fructose phosphorylating activity. *A. niger* hexokinase has now been purified and characterised, as well. It behaves like a classic hexokinase, *e.g.* catalysing phosphorylation of glucose, fructose and mannose *(21)*. *A. niger* transformants either overproducing glucokinase or hexokinase are now available for analysis in citric acid fermentations.

Overexpression of phosphofructokinase and pyruvate kinase

Recently, we have studied in our laboratory the effects of overproduction of two glycolytic enzymes, phosphofructokinase and pyruvate kinase (Fig. 1) on citric acid production by *A. niger (22)*. Moderate overexpression of these glycolytic enzymes (3 to 5-fold the wild-type level), either individually or simultaneously, did not increase citric acid production by the fungus significantly (Fig. 2). In addition, product yields [m (citric acid formed)/m (glucose consumed)] of the transformants were comparable to a wild-type strain making changes in by-product formation unlikely. Thus, phosphofructokinase and pyruvate kinase do not seem to contribute in a major way to flux control of the metabolism involved in the conversion of glucose to citric acid. The cells might compensate overexpression of phosphofructokinase and pyruvate kinase by decreasing the activity of other enzymes in the pathway. However, there were no

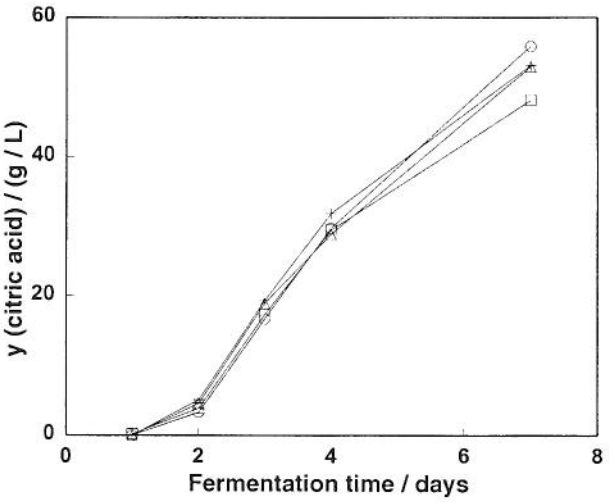

Fig. 2. Citric acid production by *A. niger* wild-type strain NW131 (+) and transformants overproducing phosphofructokinase; 49-11 (Δ), pyruvate kinase; 48-5 (□) or phosphofructokinase and pyruvate kinase; 50-2-12 (O). Strains were cultured on a medium optimised for citric acid production as described in reference *(13)*.

significant changes in the activities of other enzymes in the pathway.

Upon genetically modifying a strain, concentrations of intermediary metabolites might, as a consequence, change. Therefore, we have developed a new method to determine intracellular metabolites in *A. niger (23)*. One of the features of this method is that it ensures rapid quenching of metabolism to prevent changes in metabolite pools during sampling. Overproduction of phosphofructokinase and pyruvate kinase did not change the concentrations of intermediary metabolites. However, in strains overexpressing phosphofructokinase the level of fructose-2,6-bisphosphate, a positive allosteric effector of phosphofructokinase, was reduced almost two-fold compared with the wild-type strain. Measurements with purified phosphofructokinase, using substrate, product and effector concentrations found intracellularly, showed that such a reduction in the fructose-2,6-bisphosphate level could decrease the specific activity of phosphofructokinase in the cell significantly. Thus, the fungus seems to adapt to overexpression of phosphofructokinase by decreasing the specific activity of the enzyme through a reduction in the level of fructose-2,6-bisphosphate.

Conclusions and prospects

Genetic engineering of *A. niger* to improve citric acid production has only just been initiated. However, there are many challenges. There is a strong need for a proper characterisation of *A. niger* primary metabolism which will enable quantitative analysis of metabolism to pinpoint important steps in the metabolic pathway. In addition, modified strains should be analysed thoroughly, since the organism may find unexpected ways to circumvent specific alterations in the metabolism. With the present availability of quite a number of relevant *A. niger* genes, we think metabolic engineering is a promising approach.

Acknowledgements

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References

1. M. Röhr, C. P. Kubicek, J. Kominek, Citric Acid. In: *Biotechnology, Vol. 3*, H.-J. Rehm, G. Reed (Eds.), Verlag Chemie, Weinheim (1983) pp. 419-454.
2. C. P. Kubicek, M. Röhr, *CRC Crit. Rev. Biotechnol. 3* (1986) 331.
3. M. Mattey, *CRC Crit. Rev. Biotechnol. 12* (1992) 87.
4. C. F. B. Witteveen, M. Veenhuis, J. Visser, *Appl. Environ. Microbiol. 58* (1992) 1190.
5. C. F. B. Witteveen, P. Van de Vondervoort, K. Swart, J. Visser, *Appl. Microbiol. Biotechnol. 33* (1990) 682.
6. C. P. Kubicek, G. Schreferl-Kunar, W. Wöhrer, M. Röhr, *Appl. Environ Microbiol. 54* (1988) 633.
7. C. F. B. Witteveen, J. Visser, *FEMS Microbiol. Lett. 134* (1995) 57.
8. N. C. Van de Merbel, G. J. G. Ruijter, H. Lingeman, U. A. Th. Brinkman, J. Visser, *Appl. Microbiol. Biotechnol. 41* (1994) 658.
9. M. Röhr, C. P. Kubicek, O. Zehentgruber, R. Orthofer, *Appl. Microbiol. Biotechnol. 27* (1987) 235.
10. D. B. Kell, H.V. Westerhoff, *FEMS Microbiol. Rev. 39* (1986) 305.
11. D. B. Kell, H.V. Westerhoff, *Trends Biotechnol. 4* (1986) 137.
12. H.V. Westerhoff, *Trends Biotechnol. 13* (1995) 242.
13. N.V. Torres, *Biotechnol. Bioeng. 44* (1994) 104.
14. N.V. Torres, *Biotechnol. Bioeng. 44* (1994) 112.
15. G. J. G. Ruijter, J. L. Snoep, J. Visser, Modelling of carbohydrate metabolism in citric acid producing *Aspergillus niger*. In: *BioThermoKinetics of the Living Cell*, H. V. Westerhoff, J. L. Snoep, F. E. Sluse, J. E. Wijker, B. N. Kholodenko (Eds.), BioThermoKinetics Press, Amsterdam (1996) pp. 413-416.
16. N. V. Torres, E. O. Voit, C. González-Alcón, *Biotechnol. Bioeng. 49* (1996) 247.
17. F. Steinböck, S. Choojun, I. Held, M. Röhr, C. P. Kubicek, *Biochim. Biophys. Acta, 1200* (1994) 215.
18. H. Panneman, G. J. G. Ruijter, H. C. Van den Broeck, E. T. M. Driever, J. Visser, *Eur. J. Biochem. 240* (1996) 518.
19. M. A. Blázquez, R. Lagunas, C. Gancedo, J. M. Gancedo, *FEBS Lett. 329* (1993) 51.
20. G. J. G. Ruijter, H. Panneman, H. C. Van den Broeck, J. M. Bennett, J. Visser, *FEMS Microbiol. Lett. 139* (1996) 223.
21. S. P. Colowick, The hexokinases. In: *The Enzymes, Vol. IX B*, P. D. Boyer (Ed.), Academic Press, New York (1973) pp. 1-48.
22. G. J. G. Ruijter, H. Panneman, J. Visser, *Biochim. Biophys. Acta, 1334* (1997) 317.
23. G. J. G. Ruijter, J. Visser, *J. Microbiol. Meth. 25* (1996) 295.

Metaboličko inženjerstvo glikolitičkog puta u *Aspergillus niger*

Sažetak

Limunska se kiselina proizvodi vrenjem koristeći filamentoznu gljivu Aspergillus niger. Biosintetski put pretvorbe šećera, kao supstrata, u limunsku kiselinu obuhvaća glikolizu i dio trikarboksilnog ciklusa. U radu su navedena novija otkrića u poboljšanju djelotvornosti sojeva A. niger postignuta genetičkim inženjeringom. Razmatrane su značajke primarnog metabolizma A. niger te prekomjerna ekspresija gena za glikolizu u A. niger, uz dodatnu analizu proizvodnje limunske kiseline s pomoću modificiranih sojeva.

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Citric Acid Production from Rape Seed Oil by *Aspergillus niger*

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Summary

Various factors affecting culture conditions and citric acid production from rape seed oil were studied using a mutant strain of Aspergillus niger. It was found that citric acid was produced with a good yield from 97% in shaking culture to about 115% in a jar-fermenter, calculated against oil. Beside the property of used strain, which is capable of high citrate accumulation, the important factor in this process had a C:N:P ratio of 3:0.03: 0.008. A considerable delay of conidia germination was the most unfavourable effect extending the time course of fermentation. Citric acid was the only organic acid detected in fermentation liquor.

Keywords: citric acid fermentation, *Aspergillus niger*, rape seed oil

Introduction

Since the seventies, fermentative production of citric acid from lipids, mainly from natural oils was studied using yeasts. The most efficient producers were mutant strains of Candida lipolytica (1–5). Citric acid was produced with a good yield from about 50 to above 140% calculated in relation to oil. In general, however, d-isocitric acid, as a by-product, was accumulated, which makes sometimes above 50% of total acids.

My own preliminary study showed that natural oil and fatty acids were also metabolized by *Aspergillus niger* and can function as a sole carbon source for growth and citric acid production (6,7).

In this report various factors affecting citrate production from crude low erucic rape seed oil by *Aspergillus niger* were described.

Materials and Methods

Microorganism

A mutant strain *Aspergillus niger* IBR-1-25 was used. It was obtained by twice mutagenic treatment with UV-rays of conidia from *Aspergillus niger* KBR (8).

Culture conditions

Submerged cultures were carried out in a water-bath reciprocal shaker and in a stainless steel jar-fermenter.

The culture medium was prepared in distilled water. It consists of oil and mineral salts such as: NH_4NO_3; KH_2PO_4; $MgSO_4 \cdot 7H_2O$; $FeCl_3$; $CuSO_4 \cdot 5H_2O$; $ZnSO_4 \cdot 7H_2O$; $MnCl_2 \cdot 7H_2O$ and $Na_2MoO_4 \cdot 2H_2O$. Organisms were grown in 500 mL conical flasks containing 100 mL of medium on the shaker.

All experiments were run in the presence of 40 g/L of oil and the initial concentration of mineral salts, inoculum size, pH value and culture temperature were tested. As a result of these investigations the following composition of medium was defined: 40 g of oil; 0.857 g of NH_4NO_3; 0.35 g of KH_2PO_4; 0.3 g of $MgSO_4 \cdot 7H_2O$; 400 μg of Fe^{2+} and 200 μg of Cu^{2+} per 1 L of the solution. The most profitable inoculum size was 10^5/mL, pH value 3.5 and culture temperature about 30 °C.

When the jar-fermenter of 6 L was used the volume of medium was 4 L and the initial oil concentration, air-flow rate and agitation speed on citric acid yield were estimated.

Analytical methods

Total acidity of culture broth was determined by titration with 0.05 M of NaOH using phenolphtalein as indicator and expressed as anhydrous citric acid.

Separated mycelium from fermentation mash was washed by ethyl ether and weighed after drying.

Residual oil after exhaustion from samples and evaporation of ethyl ether was dried and weighed as before.

Concentration of fatty acids methyl esters was determined by gas chromatography.

The homogenity of fermentation was estimated using qualitative TLC chromatography *(9,10)* the pentabromoacetone method *(11)* and enzymatic method *(12)*.

Results and Discussion

Experiments in shaking culture

Table 1 shows the essential chemical composition of the raw material. It can be seen that used oil makes a wealthy source of carbon in excellent majority in the form of unsaturated fatty acids. Moreover a low contents of metals was confirmed.

In research concerning initial concentration of nitrogen and phosphorus, NH_4NO_3 was used as the N source. The whole nitrogen content was taken into consideration. The earlier studies showed that among several N sources ammonium nitrate was the most profitable for citrate production *(13)*.

The initial concentration of NH_4NO_3 was tested in the range from 0.01 to 0.04 g/100 mL with respect of nitrogen, while KH_2PO_4 from 0.001 to 0.02 g/100 mL with respect of phosphorus. Addition of $MgSO_4 \cdot 7H_2O$ was always constant *i.e.* $\gamma(Mg) = 0.003$ g /100 mL. The inoculum size was of 10^5 conidia/mL of medium, pH value was adjusted to 3.0 and culture temperature maintained at 30 °C.

The dependence of N and P concentrations in relation to weight of carbon (3.0 g/100 mL added with oil) is shown in Fig. 1. It can be seen, that the nitrogen level was a real parameter influencing the citric acid production. The most adventageous concentration of nitrogen was 0.03 and phosphorus about 0.008 g/100 mL of medium. Thus as the most efficient C:N:P ratio (3 : 0.03 : 0.008) was accepted and used in the subsequent investigations.

The initial C:N:P ratio has an important meaning in the batch culture system. In the exponential growth phase a suitable nitrogen level is needed for the indispensable biomass growth. During the idiophase, nitrogen and especially phosphorus must be limited. The citric acid production will commence since a critical N and P levels are reached. The restriction of vegetative fungal growth and respiration activity favoured a high citrate accumulation.

Table 1. Chemical composition of 100 g low erucic rape seed oil

Component	m/g	Component	m/μg
Moisture and volatile compounds	0.4	Magnesium	1600
Glycerol	9.7	Ferrum	350
Whole fatty acids	88.4	Copper	200
palmitic C_{16-0}	3.1	Cadmium	5
palmitoleic C_{16-1}	0.1	Zinc	36
stearic C_{18-0}	0.8	Manganese	60
oleic C_{18-1}	49.8	Molybdenum	7
linoleic C_{18-2}	19.8	Lead	20
linolenic C_{18-3}	8.6		
eicosanoic C_{20-1}	1.9		
behenic C_{22-0}	0.6		
erucic C_{22-1}	3.6		
Carbon	74.43		
Phosphorus	0.03		

When the inoculum size was tested, spore suspension were added in amounts to give a final concentration of 10^4, 10^5, 10^6 and 10^7/mL. The highest yield of citric acid (89% against oil) was confirmed with inoculum size of 10^5/mL. These investigations showed that the spore germination was retarded by the presence of oil in the medium. It was probably caused by a thin layer of oil wich covered the surface of conidia and repressed the water penetration inside.

The indispensable time to start of conidia germination was 20–26 hours, depending on pH and temperature level (Figs. 2 and 3). At the optimal pH value (3.5) and culture temperature (30 °C), considering the citric acid production, conidia germinated after about 21 hours. In the following tests, when conidia maintained for 6 hours at 34–36 °C after inoculation and then the temperature was slowly reduced until 30 °C, the germination phase was observed after 17–19 hours. The exponential growth phase, however, commenced towards the end of the second day and maintained until 5–6 days. As a consequence the fermentation process came to the end after 12–13 days.

The influence of some trace metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+} and Mo^{2+}) on citrate accumulation was tested in the range from 0 to 200 μg/mL of medium. It was found, that only Fe^{2+} and Cu^{2+} ions had a visible influence on the fermentation process. When 40 μg Fe^{2+} and 20 μg Cu^{2+} per 100 mL were simultaneously added the yield of citric acid was about 95% calculated from oil. Zn^{2+}, Mn^{2+} and Mo^{2+} ions in concentration of about

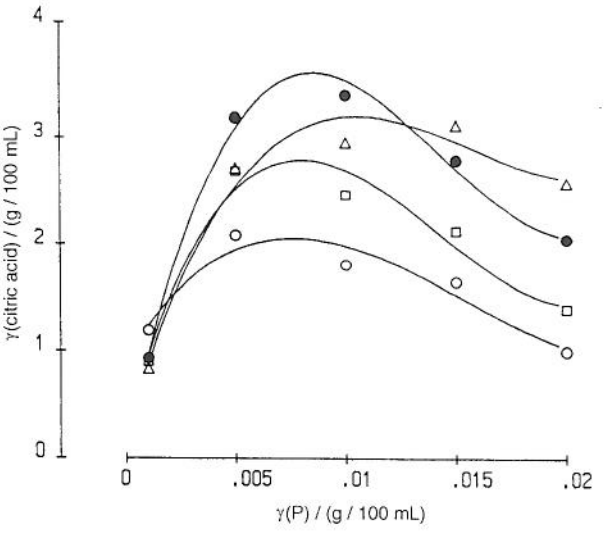

Fig. 1. Effect of initial concentration of nitrogen and phosphorus on citric acid production. Nitrogen concentration γ(N): (○) 0.01, (□) 0.02, (●) 0.03 and (Δ) 0.04 g/100 mL

30–40 μg/100 mL had not unfavourable effect on this process, however, higher concentrations repressed citrate accumulation. Fe^{3+} had no effect in the whole range of concentrations.

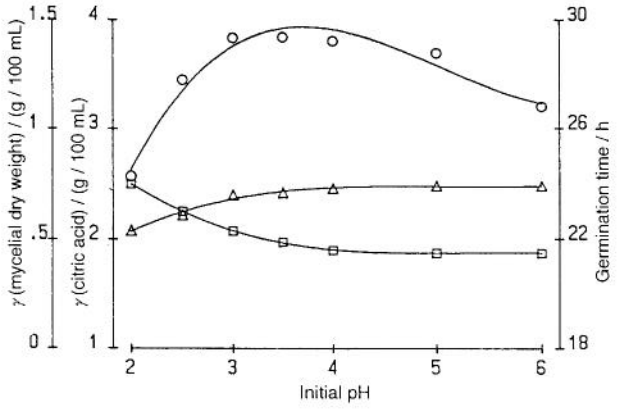

Fig. 2. Effect of initial pH value on citric acid production, mycelial growth and beginning of conidia germination: (O) citric acid, (Δ) mycelial net weight, (□) germination time

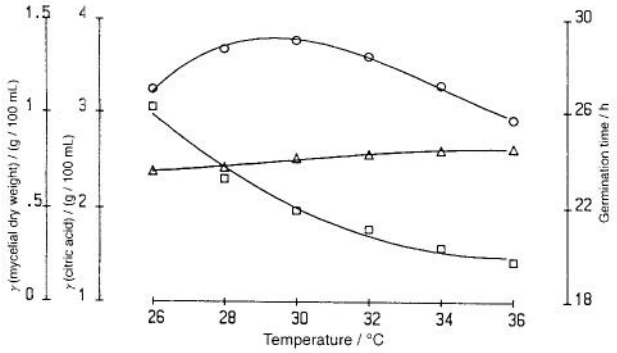

Fig. 3. Effect of culture temperature on citric acid production, mycelial growth and beginning of conidia germination: (O) citric acid, (Δ) mycelial net weight, (□) germination time

Investigation with jar-fermenter used

At first, using the optimal C:N:P ratio the initial concentration of substrate was optimized. The concentrations of 4, 6, 8, 12 and 14 g/100 mL of rape seed oil was used alternately. Mineral compounds were added proportionaly to the oil contents. Inoculum size, pH value and temperature were the same as in the shaking culture. Fermentations were run at a constant rate of aeration (1.4 v/v/min) and impeller speed (100 r.p.m.).

It was shown that optimal nutrient conditions and more profitable aeration of medium favoured the biomass formation, product accumulation and shortened the fermentation time (Table 2). The highest concentration of citric acid was detected in the presence of 6 and 8% of oil, *i.e.* 6.37 and 8.67 g per 100 mL. Thus the yield of fermentation was about 106 and 108%. Even in the case when 8% of oil was added the fermentation process was notably shorter in comparison with shaking culture containing 4% of substrate in the medium. In further experiments 8% of oil was added.

Subsequently the influence of aeration and agitation of culture medium on citrate accumulation was examined. It was found, however, that the oxygen sensor was soon covered by an oil membrane and made dissolved oxygen tension measurement impossible. Thus the experiments were limited to determination of air-flow rate and agitation speed. As shown in Table 3 both parameters affected citric acid production and the most profitable magnitudes were 1.0 v/v/min of aeration rate and 860 r.p.m. of impeller speed. Too small volume of air reduced the mycelial growth and citric acid production, whereas, excessive increase of both parameters intensified mycelial growth but decreased the yield of fermentation.

Table 3. Effect of air-flow rate and agitation speed on citric acid yield (g/g) in presence of 8% oil

Agitation speed/ r.p.m.	Air-flow rate v/v/min			
	0.66	0.83	1.0	1.4
650	0.69	0.86	0.95	0.89
860	0.90	1.08	1.12	1.04
1000	–	0.94	1.01	0.92

Table 4. Utilization of fatty acids during fermentation with 8% oil in the medium

Fatty acids	Initial concentration	Utilized fatty acids	
	g/100 mL	g/100 mL	%
Whole fatty acids	6.96	6.38	91.7
palmitic C_{16-0}	0.20	0.20	100.0
palmitoleic C_{16-1}	trace	–	–
stearic C_{18-0}	0.05	0.04	80.0
oleic C_{18-1}	3.96	3.96	93.2
linoleic C_{18-2}	1.56	1.51	94.9
linolenic C_{18-3}	0.71	0.68	95.8
eicosanic C_{20-1}	0.14	0.08	57.1
behenic C_{22-0}	0.03	0.02	66.6
erucic C_{22-1}	0.31	0.16	51.6

Table 2. Effect of initial oil concentration on citric acid production, mycelial growth and fermentation time

Oil content	Citric acid	Yield against oil	Mycelial dry weight	Substrate utilization	Fermentation time
% (w/v)	g/100 mL	%	g/100 mL	%	days
4	0.99	99.0	0.91	93.3	8
6	6.37	106.2	1.39	92.8	8–9
8	8.67	108.4	1.94	92.1	9
10	0.89	89.3	2.42	88.8	10–11
12	0.76	74.4	2.89	87.1	11–12

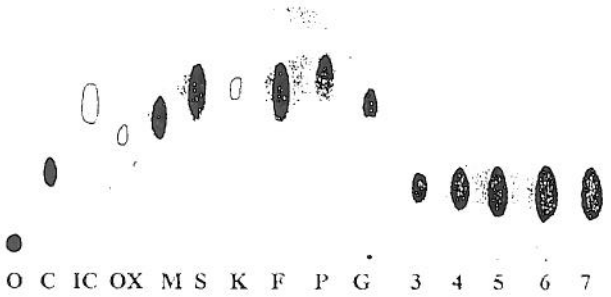

Fig. 4. TLC Chromatogram of organic acids accumulated during fermentation in presence of 8% oil.
Standard acids: O-oxalic, C-citric, IC-isocitric, OX-oxalacetic, M-malic, S-succinic, K-α-ketoglutaric, F-fumaric, P-pyruvic, G-gluconic. 3–7: citric acid accumulated during fermentation

At last the fermentation was carried out considering all defined parameters of culture conditions and the inoculum size was enlarged to $5 \cdot 10^5$ per mL. After nine days 9.16 g of citric acid in 100 mL of medium was detected, and the yield of fermentation was about 114.5% calculated from oil.

The performed investigations showed that the substrate was not entirely consumed. Increasing of initial oil concentration caused decreasing of its utilization.

As the result of glyceride digestion by fungal lipases, fatty acids and glycerol were released and both of them could be assimilated. To investigate to which extent all fatty acids were utilized the consumption of fatty acids was studied. The contents of fatty acids in fresh oil and in residual oil was determined by gas chromatography. Utilization degree of fatty acids during the fermentation run was defined by comparison of both results. The final utilization of fatty acids is shown in Table 4. As it can be seen all fatty acids were consumed but in different range. The joint consumption of three fundamental acids (oleic, linoleic and linolenic) compared with total acids utilization was over 92%. The lowest utilization concerned the erucic acid. The utilization of glycerol during fermentation process was not estimated.

The homogenity of fermentation was also studied. Quantitative analysis for organic acids with TLC chromatography showed that only citric acid was accumulated (Fig. 4). Moreover considerable testing was made with the purpose of isocitrate detection. It was done with two methods and no d-isocitric acid was detected.

Conclusions

The rape seed oil is a wealthy source of carbon, which contents is doubled in relation to carbohydrate materials.

The used oil is an efficient substrate for citric acid production. There is a real possibility to obtain a high yield of fermentation which was already shown in this work.

The fermentation process displayed a high degree of homogenity.

There were also found some negative events taking place, like very late spore germination, prolongated time of fermentation process and incomplete substrate utilization.

References

1. Y. Ikeno, M. Masuda, K. Tanno, I. Oomori, N. Takanishi, *J. Ferment. Technol. 53* (1975) 752.
2. M. Masuda, Y. Ikeno, Y. Matsumoto, *Japan Kokai, 7525789* (1975).
3. R. Yamada, K. Tanno, Y. Ikeno, *Japan Kokai, 7553585* (1975).
4. Showa Oil Co Ltd., *Japan Kokai, 6024191 (1985).*
5. T. Matsumoto, Y. Ichikawa, *Japan Kokai, 612 19392* (1986).
6. E. Elimer, *Prace Naukowe AE Wroclaw, Technologia, 291* (1984) 15.
7. E. Elimer, *Przem. Ferment. i Owocowo-Warzywny, 29* (1985) 24.
8. E. Elimer, *Prace Naukowe AE Wroclaw, Technologia, 476* (1989) 49.
9. E. Stahl: *Dünnschichtchromatographie*, Springer – Verlag, Berlin, Heidelberg, New York (1967).
10. E. Elimer, *Prace Naukowe AE Wroclaw, Technologia, 313* (1985) 47.
11. E. Stern, *Methods Enzymol. 3* (1967) 426.
12. A. Kornberg, W. E. Pricer, *J. Biol. Chem. 189* (1951) 123.
13. E. Elimer, *Prace Naukowe AE Wroclaw, Technologia, 397* (1987) 78.

Proizvodnja limunske kiseline iz repičinog ulja s pomoću *Aspergillus niger*

Sažetak

Koristeći mutantne sojeve A. niger, proučavani su razni faktori koji utječu na uvjete uzgoja i proizvodnju limunske kiseline iz repičinog ulja. Utvrđeno je da je iskorištenje limunske kiseline bilo 97% u tresilici, a oko 115% u fermentoru, računajući na količinu ulja. Odnos C : N: P = 3 : 0,03 : 0,008 bio je bitan za proizvodnju limunske kiseline. Usporeno pupanje konidija najnepovoljnije je djelovalo produžujući proces vrenja. Limunska kiselina bila je jedina organska kiselina utvrđena u fermentacijskoj podlozi.