Original scientific paper
UDC 547.477.057:582.282.123

ADVANCED BIOTECHNOLOGY FOR CITRIC ACID AND CITRIC ACID ENZYMES

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Abstract

Today there is a world tendency to use starch containing raw materials for citric acid production instead of the traditional ones. An enzymes-induced synthesis acting as a catalyst for the polysaccharide hydrolysis in the acidic media occurs during fermentation together with the main product. The goal of this research is to develop technologies for the production of several desired products during a one process.

Hydrolyzed corn starch was used as a raw material. The following devices were applied: a shaking incubator Multitron, a drum set Sartorius, cartridge filters with molecular weight cut-off of 100 and 15 kDa for citric acid and enzymes separation, extraction and purification. The enzyme activity was evaluated by spectrometry. Methods to regulate citric acid biosynthesis during corn starch hydrolysates fermentation using *Aspergillus niger* strains are developed.

It was established that glucose concentration has a main influence on the citric acid biosynthesis. Enzyme production depends largely on the maltose concentration. A considerable influence exerts the carbohydrate - nitrogen ratio being C:N = 55:1. Due to the optimal fermentation conditions balance mechanisms to produce two microbial biosynthesis products are assured. Finally a basis for the new integrated technology that permits to produce citric acid and a number of acid-stable enzymes in one process is developed. The quality of the isolated citric acid satisfies GOST R 908-2004. An integrated enzymatic formulation with α-amylase, glucoamylase, protease, xylanase, maltase activities is received together with the citric acid. The amylase activity corresponds to foreign multi-enzyme preparations. The isolated enzymes formed part of the multipurpose complex food additive destined to intensify fermentation processes and to improve final food product organoleptic properties.

The new technology is able to meet world market competition. It is possible to apply the isolated substances as a part of food additives for produce bread, bier, alcohol and soft drinks and as a processing aid in starch, light and medical industries.

Key words*: Citric acid, Acid-stable amylolytic enzymes, Multipurpose food additives.*

1. Introduction

Citric acid (E330) is a well-known acidifier, acidity regulator, antioxidant and sequestrant [1]. Its consumption increases every year and is up to 440 million tons in the European market. Citric acid is produced by microbial fermentation. Micromycete *Aspergillus niger* strains are normally used as the producer. For the first time citric acid was produced biotechnologically by Wehmer cultivating Penicillium *glaucum* in 1893. As a commercial product it was produced first from Italian lemons in England at the beginning of the 19th century. It was in 1917 when the American scientist Currie found that a considerable amount of citric acid accumulates at the fermentation of the saccharose mineral medium by *Aspergillus niger* micromycete. As a result of this discovery Pfizer Inc. founded citric acid production in the United States of America in 1923, and since 1929 Citrique Belge started to produce it as well. At the end of the 19th century Grimoux and Adams succeeded in effecting the chemical synthesis of citric acid from glycerin and later from acetone dichloride. Although the initial material is accessible this method is non-competitive in comparison with the microbiological one mainly because of the disequilibrium between raw material and final product prices. Later it was discovered that other *Aspergillus* genus micromycetes are able to synthesize citric acid [2].

Today published data shows the ability of various yeasts (*Yarrowia*, *Candida*) to produce citric acid if cultivated with ethanol and rapeseed oil [3, and 4]. Synthesis of large amounts of the byproduct isocitric acid is a negative factor of this method.

Although there are many microorganisms able to synthesize citric acid effectively, *Aspergillus niger* has a number of advantages being tolerant to large concentrations of carbohydrates in the nutrient medium, resistant to fluctuations of fermentation medium pH and temperature [5].

Various carbohydrate-containing natural substrates are used as a carbohydrate source for citric acid biosynthesis in the world. They are, for example, glucose, molasses, oilseed residues, oil meal, cores, beet pulp, haulms, straw of agricultural cultures, potato fibers, grain and potato slops, saw dusts; wood, flour and starch hydrolysates [6, 7, and 8].

Presently there is a worldwide tendency for citric acid production factories to change over to a new raw material - starches. Their benefit is high sugar content; during fermentation these sugars transform into citric acid. Starches are practically free from iron salts and ashy elements, including calcium salts that negatively influence the properties and activity of the producer.

Starch hydrolysis products induce the synthesis of amylolytic enzymes. Representatives of *Aspergillus niger* are often occurring among the amylase producers [9, 10, and 11]. To intensify the technological process, amylases are widely used in the food and processing industry as biocatalysts for raw material hydrolysis. Acid-stable amylases which catalyze polysaccharide hydrolysis in the acid medium are of special interest. The world enzymes market increases annually at an average of 10%, and the European market, by virtue of innovative development, at 3.5%.

Starch hydrolysates can be considered as an effective raw material for the production of several products of the microbial synthesis in one biotechnological process as they can be used to produce citric acid and amylases by *Aspergillus* [1]. The goal of the research is to develop unified technologies for the production of several target products, citric acid (E330) and acid-stable enzymes, in one process.

2. Materials and Methods

Citric acid producer strain *Aspergillus niger* L-4 was used to perform this work. It was produced by mutagenesis and selected for citric acid biosynthesis during molasses and/or sugar fermentation. The raw material was corn starch [12]. The fed-batch fermentation in 750 mL shake flasks was applied.

The fermentation was carried out by batch method using Multitron shaking incubator. The nutrient solution volume was (50 ± 2) cm^3; the temperature was (33 ± 1) ^0C at the vegetative seed mycelium growth stage and (31 ± 1) ^0C during fermentation. The stirring rate was (200 ± 10) rev/min.

Hydrolyzed starch sugar content was estimated by the dextrose equivalent (DE) value which defines total glucose and maltose content in terms of dry substances.

Ammonium salts, urea, dry fermentative peptone for bacteriological purposes, yeast extract and autolysing mycelium of *Aspergillus niger* were used as nitrogen sources. Salts of mineral acids (phosphoric, hydrochloric, nitric and sulfuric) were used as sources for phosphorus, sulfur, micro and macro elements.

The optimum nutrient composition for producer's cultivation at citric acid and amylase production is: g/dm^3: hydrolyzed corn starch with dextrose equivalent DE = (25 ± 1)% - 20; NH_4NO_3 - 2,1; $MgSO_4{\cdot}7H_2O$ - 25; KH_2PO_4 - 0,16; $ZnSO_4{\cdot}7H_2O$ - 0,5; $CuSO_4{\cdot}5H_2O$ - 0,20; $FeSO_4{\cdot}7H_2O$ - 0,005; mycelial autolysate - 0,4; water - up to 1 L, medium pH = 6 [13].

Citric acid and enzymes were separated from liquid culture by baromembrane filtration method. Microfiltration was performed with the use of cartridge filters with the propylene membrane pore sizes of 1.2 µm and 0.65 µm ("Sartopure PP") and 0.45/0.20 µm for a polyethersulphone pore ("Sartobran"). Polysulphon-base hollow fiber membrane modules, which separate substances with a molecular mass of 100 kDa and 15 kDa, were used for ultrafiltration. For baromembrane filtration, the operating pressure was 0.2 MPa and the temperature - 25 ^0C, the solution throughput rate 50 cm^3/min.

Enzyme catalytic effectiveness was estimated by spectrophotometry and the activity of α-amylase, glucoamylase, maltose, protein, xylanase, saccharification and dextrin were determined.

The enzyme quantity catalyzing 1 g starch hydrolysis to dextrins with various molecular weight at 30^oC and pH = 4.7 during 1 h was taken as a α-amylase activity measuring unit; the enzyme quantity releasing glucose 1 mmol at 1g starch hydrolysis at 30 ^0C and pH = 4.7 during 1 min. was taken as a glucoamylase activity measuring unit; the enzyme quantity splitting 1g maltose to glucose to 30% at 30 ^0C and pH = 4.7 during 1 h was taken as maltose activity measuring unit; the enzyme quantity catalyzing 1 g hemoglobin to 50% at 30 ^0C and pH = 2.34 during 30 min. was taken as proteolytic activity measuring unit; the enzyme quantity catalyzing 1 g xylan to 1 mg reducing hydrocarbon calculated as xylose at 40 ^0C and pH = 4.8 during 1 h was taken as xylanase activity measuring unit; the enzyme quantity catalyzing 1 g starch at 30 ^0C and pH = 4.7 during 1 h to reducing hydrocarbons which are 20% from starch quantity was taken as saccharifying activity measuring unit; the enzyme quantity catalyzing 1 g dextrin to reducing sugars at 30 ^0C and pH = 4.8 during 1 h which are 30% from dextrin quantity was taken as xylanase activity measuring unit. The whole analysis was carried out three time and the data are expressed as average ± SE (standard error).

3. Results and Discussion

The results of the research showed that *Aspergillus niger* L-4 synthesizes only a trace amount of amylase

using molasses-adapted medium. The amylase quantity increases with the change in the qualitative carbon sources composition. Sucrose is the main carbohydrate component of molasses. Replacing molasses with hydrolyzed starch containing dextrins, maltose and glucose, α-amylase and glucoamylase increases activity significantly. Alpha amylase activity in the producer's cell reaches 2.7 - 3.0 units (U) calculated as 1 g of dry biomass (DB), glucoamylase activity is about 10 - 15 units per 1 g DB. Alpha amylase activity in the native solution varies from 0.17 to 0.26 U/cm^3, glucoamylase activity varies from 3.2 to 4.3 U/cm^3. These results are the basic level for low molecular sugars. If the sugar structure is more complex, e.g. on starch hydrolysates, this level increases several times. The same results are known for micromycete *Aspergillus kawachii* widely spread for sake production being an active acid-stable amylase producer at the solid-substrate fermentation [1]. It means that the type of strain of *Aspergillus* can vary the metabolism direction. A medium does not interfere with the genetic code of the cell but influences the phenotypic gene expression.

The vegetative seed mycelium activity depends, largely, on its age at the moment when it is transferred into the fermentation medium. According to the results of the study, 48 h cultivation showed the best acid-forming capacity for the seed mycelium strain of *Aspergillus niger* L-4. The highest fermentative activity exerts in a wide age range (36 - 48 h). It is determined that the conidium suspension quantity has a greater effect on amylase activity than on citric acid formation. The optimal conidium suspension proportion of 7 x 10^7 cells/cm^3 is 20% of nutrient medium volume.

The corresponding gene presence is not sufficient to form enzymes in a significant quantity. The presence ("induction") or absence ("repression") of catalyzing substances related to the reaction is needed. Regarding citric acid biosynthesis, it is very important to regulate this process that affects the activity of amylolytic enzymes of the *Aspergillus niger* L-4 producer. Introducing carbohydrates that are specific to the synthesized enzyme into the nutrient medium is one of the ways of such influence. The hydrolyzed starch consists of dextrins and maltose which in certain concentrations induce the amylase biosynthesis.

Specific substrates cause enzymes accumulation in the cell to destroy the respective substrate. The forming low sugar compounds can enter the cell and induce this enzyme biosynthesis. The investigation results shown that the amylase activity synthesized by *Aspergillus niger* L-4 to the greatest extent depends on maltose quantity. Maltose was identified in the culture medium throughout the whole biotechnological process. Under its own glucoamylase *Aspergillus* action, maltose was hydrolyzed to glucose, essential for the producer growth and development. The maltose identified in the culture medium was formed as the result of its own α-amylase producer's action on dextrins. The initial maltose content that favors amylase active synthesis is 75 - 80 g/dm^3. Their fermentative activity was 0.5 - 1.8 U/cm^3 for α-amylase, and 15 - 20 U/cm^3 - for glucoamylase.

The enzymes activity varied according to the carbohydrate concentration ratio in the hydrolyzed starch. The most productive citric acid biosynthesis by the strain *Aspergillus niger* L-4 occurs at the glucose, maltose and dextrins concentration ratio in the nutrient medium of 1 : 6 : 49 (DE = 20%) [14]. It was established that citric acid and amylases are produced more actively due to maltose duplication and dextrin decrease by 1.2 - 1.3 times (DE - 25%). The total sugar concentration in the medium was about (130 - 140 g/dm^3). In these conditions the catabolite repression, typical for the most filamentous fungi, can be deleted [5, 15]. Cultivation of the strain *Aspergillus niger* L-4 in the medium saturated with glucose and maltose can become more complicated because of the amylase synthesis repression. The study results showed that there is no glucose in the medium during the active amylase biosynthesis (third day of the fermentation). For this reason there should not be amylase catabolite repression. The strain produces α-amylase proportionally to maltose concentration increase in the medium. We suppose that it is not linked only with the maximum induction of this enzyme synthesis but because there is no catabolite repression influence as well. Another conclusion was drawn in case of glucoamylase. It was based on comparing data obtained at the hydrolyzed starch fermentation with DE = 20% and DE = 25%. Only maltose was present in the medium at the active amylase biosynthesis. For the efficient citric acid biosynthesis maltose content (DE = 20%) is 4.5 times higher than in conditions which ensure amylase biosynthesis intensification (DE = 25%). The obtained data analysis confirms the presence of the glucoamylase catabolite repression at the directed citric acid biosynthesis.

The necessary condition for the efficient citric acid biosynthesis is to limit nitrogen and phosphorous concentration in the nutrient medium. At the hydrolyzed starch fermentation the preferred ratio for citric acid biosynthesis is C : N = 75 : 1 and N : P = 13.5 : 1 [5]. As the result of the study it is determined that at the carbohydrate and nitrogen ratio of C : N = 30 : 1, mycelium growth and development of the strain *Aspergillus niger* L-4 retreat. The DB value was (5.3 ± 0.5) g/dm3. The elevated carbohydrate content (by a factor of 75) in comparison with nitrogen leaded to active biomass accumulation and fermentative system activity; as the acid-forming capacity of the producer decreased. Using NH_4NO_3 the α-amylase and glucoamylase activity increased at C : N = 55 : 1. It is noted that hydrolyzed mixture of corn starch and corn flour (N : P = 8 : 1)

considerably influence the amylase biosynthesis. The most active amylase biosynthesis was under the presence of the mycelial mass autolysate (N : P = 6.7 : 1).

It was substantiated that it is necessary to use microelements in the form of salts $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 and microelements Zn, Cu together with Fe within sulfuric acid salts to increase amylase production activity by the strain L-4. Mineral and organic nitrogen sources and microelements used together make it possible to increase amylase activity by a factor of 1.3 - 3.3 in comparison to media fermentation where NH_4NO_3 was used only as a nitrogen source. The index of sugar conversion into citric acid was at the control level (C : N = 75 : 1).

Based on the obtained data, several ways to regulate citric acid and amylase biosynthesis were determined:

- keeping constant ratio level for medium carbohydrates concentrations (dextrins, maltose, glucose);
- ensuring the C : N and N : P ratio in nutrient medium is at the requited level to activate target product biosynthesis;
- superinducing an organic nitrogen source which helps to increase enzymes activity at the effective acidogenesis into the nutrient medium.

The native solution properties are shown in the Table 1.

Table 1. Native solution properties

Item	Index value
Color index D_{360}, AU	2.5 ± 0.1
Cloud number D_{760}, AU	1.1 ± 0.1
pH	2.0 ± 0.1
Citric acid mass concentration, g/dm^3	109 ± 5
Gluconic acid mass concentration, g/dm^3	6.9 ± 0.1
Oxalic acid mass concentration, g/dm^3	none
Ash mass concentration, g/dm^3	0.30 ± 0.02
Sugar mass concentration, g/dm^3	2.1 ± 0.1
Protein mass concentration, g/dm^3	4.3 ± 0.1
Colloidal mass concentration, g/dm^3	2.0 ± 0.1
Fermentative activity, u/cm^3: - α-amylase - glucoamylase	 3.8 ± 0.1 141 ± 2

The native solution study showed that they are colored, cloudy liquids with citric acid, enzymes, residual sugars, mineral and protein substances. The native solutions pigment is caused by the content of melanins and melanoidines. They are nitrogen-containing compounds which favor carbohydrates interaction with amino acids and peptides.

Several methods to purify native solutions were studied: sorption and ion exchange, application of mineral and organic precipitators, baromembrane methods. The research results showed that for preliminary purification of native solutions it is reasonable to use bentonite and clarifying sorbent or to perform the ion exchange process by anionite (sorption at pH = 3). As a result, solutions discolored by 90% with dry matters in the quantity of 10 - 12% were obtained. The citric acid concentration in them was at the initial native solution level and the α-amylase and glucoamylase specific activity increased by 3 - 4 times. However, it became possible to combine purification of the native solution and separation of the desired substances only if microfiltration and ultrafiltration were used sequentially.

The obtained ultrafiltrate was clarified by activated charcoal and the solution was concentrated up to the density of (1265 ± 5) kg/m^3. The citric acid yield (50% solution) was 92.8 - 93.1%. Regimes for citric acid crystallization were studied. The citric acid crystals (0.05%) as a fuse were added into the crystallizing solution under +37 ^{0}C. The crystallization was performed step by step with the solution cooling up to +27 ^{0}C, with the rate of 10 ^{0}C and then up to + 22 ^{0}C with the rate of 3 ^{0}C/h [1]. The isolated crystals were dried at + 50 ^{0}C. As a result, citric acid in crystal form complying with the requirements of Russian technical standard GOST 908-2004 and Codex Alimentarius standard 192 with the yield of (78 ± 2)% was produced [6]. The concentrate obtained after the ultrafiltration was viscous stringy colored solution rich in protein and dry matters (19 - 25%). The citric acid rest in it was 1.2%. The fermentative activity in the concentrate was at the level of (280 ± 20) U/cm^3 for α-amylase and (6500 ± 500) U/cm^3 for glucoamylase. The concentrate contains enzymes with maltose, proteinase and xylanase activity as well. The quantitative enzyme preparation yield was 14 - 16 g from 1 dm^3 of the native solution. The powder preparation was obtained by freeze-drying with the yield of 3 - 5 g from 1 dm^3 of the native solution. The isolated enzymes are competitive by their quality of performance with those produced by the enzyme industry abroad.

The isolated enzymes were used as a part of a new complex food additive. This additive contains acid-stable enzymes, calcium citrate, citric and lactic acids. The additive enzyme activity is shown in the Table 2.

Table 2. Complex food additive catalytic properties

Item	**Fermentative activity, units/cm^3 (liquid form); units/g (powder form)**	
	Liquid form	Powder form
α-amylase	220 ± 15	1300 ± 60
Glucoamylase	8200 ± 250	10000 ± 520
Maltade	8.5 ± 0.5	28 ± 2
Saccharifying	8000 ± 220	8200 ± 230
Dextrin	70 ± 5	120 ± 5
Protease	20 ± 2	50 ± 5
Xylanase	45 ± 5	100 ± 25

The research results showed that the quantity of reducing substances in wheat bread increases if the additive is used while baking. Gas evolution intensifies; the end product quality improves (the porosity increases by 4 - 15%, the specific volume - by 6 - 12%). The compressibility index of bread crumbs in test samples after 48 - 96 h storage was 16 - 30% higher than control level. The additive used in brewing decreases the saccharification time by 10 - 15 min., increases reducing substances in worth by 5 - 8% and beer alcohol content by 11 - 20%. It is established that this additive is effective for the malt with the fermentative hypoactivity as well as with standard characteristics. As a result, a technology to produce citric acid and acid-stable enzymes in one biotechnological process is developed. Complex enzymes used in the food additive intensify hydrolysis of carbohydrate substrates and improves end-product quality.

4. Conclusions

- New methods are developed to regulate citric acid biosynthesis during hydrolyzed corn starch fermentation using the strain of micromycete *Aspegillus niger* L-4.

- A change in the targeting process is established due to the obtained balance for carbohydrate, nitrogen and phosphoric producer nutrition.

- Balance mechanisms are provided to produce two microbial biosynthesis products - citric acid and acid-stable enzymes.

- An effective membrane method is developed to separate desired products of the microbial synthesis.

- A possibility to use isolated enzymes in food additives to produce bread, beer, alcohol and soft drinks is shown.

Acknowledgements

We would like to thank Iliana Negrutsa, PhD, for efficacious translation, design of the article and correspondence with the editors and Alexey Kargin for the linguistic revision of the article.

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