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Citric Acid Production on a Waste Starch Fraction Hydrolysate

Pridobivanje citronske kisline na hidrolizatu odpadne frakcije škroba

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Summary

The waste fraction in wheat starch production, starch B, was hydrolysed by means of liquefying and saccharifying enzymes to prepare a substrate for submerged citric acid fermentation. A suitable ***Aspergillus niger*** *strain was used. Improved yields could be obtained by growing the inoculum on a starch containing agar. The effect of trace metals in the substrate was reduced by the addition of potassium hexacyanoferrate(II).*

Povzetek

Z encimi, ki utekočinjajo in saharificirajo škrob smo hidrolizirali odpadno frakcijo pri pridobivanju pšeničnega škroba, takozvani škrob B, da bi dobili primerno gojišče za submerzno biotehnološko proizvodnjo citronske kisline. Uporabili smo primeren sev glive ***Aspergillus niger****. S pripravo vcepka na škrobnem agarju smo izboljšali dobitke citronske kisline. Vpliv elementov v sledovih v gojišču smo zmanjšali z dodatkom kalijevega heksacianoferata(II).*

Introduction

Starch B is a waste fraction obtained during production of wheat starch. It contains among others starch and protein. Therefore, it could be a valuable raw material for some production. One of the possibilities is to use this waste fraction as a carbon source in biotechnological production such as citric acid fermentation.

Citric acid has been produced biotechnologically from the beginning of this century, first by surface fermentation and after the second world war by the submerged process. Till now, *Aspergillus niger* has been almost exclusively used. Citric acid yields are strongly influenced by the composition of the fermentation medium. In most cases sucrose containing materials are used, predominantly beet and cane molasses *(1,2)*.

To our knowledge, no reference about the use of untreated starch as carbon source in citric acid production has appeared in literature.

Relatively little has been published also about the use of starch hydrolysates in citric acid fermentation *(3,4)*. Recently, the use of sweet potato starch hydrolysates for citric acid production in China was mentioned *(5)*.

One of the most serious problems in using different unrefined carbon sources is the influence of heavy metal ions which decisively decrease citric acid yields. Among them manganese has been found to be the critical metal ion *(6)*. To prevent the negative effect of metal ions most frequently potassium hexacyanoferrate(II) is added to the substrate *(7)*.

The aim of our work was to test starch B as carbon source in citric acid production.

Materials and methods

Microorganism

Aspergillus niger strain A60 from the Culture Collection of the National Institute of Chemistry, Ljubljana, Slovenia (originally strain NRRL 2270) was used throughout all experiments. It was maintained on beer wort agar slants at 30 °C. To adapt the productive strain to starch as sole carbon source it was regularly inoculated on agar slants containing gradually higher amounts of soluble starch (e.g. for the first step of the inoculum preparation the medium contained 0.5 % of soluble starch and 1.5 % of sugar in the form of beer wort, etc.). In the last step the medium composition was as follows:

Soluble starch	2.0 %
K_2HPO_4	0.35 %
KNO_3	0.5 %
$MgSO_4 \cdot 7H_2O$	1.5 %
agar	2.0 %

As inoculum for fermentation tests a water suspension of spores cultivated on media with increasing starch concentration, mentioned above, was used. The concentration of spores was 10^7 spores/mL.

Substrate

Preparation of starch hydrolysates

For all experiments wheat starch B, representing a waste material in starch production, was used.

Its composition was as follows:	
starch	65 ± 5 %
other carbohydrates (pentosanes, sugars, bran residues)	23 ± 2 %
proteins	5 ± 1 %
moisture	7 %

For liquefaction bacterial α-amylase BAN 120 L (NOVO, Denmark) and for saccharification DEXTROZYME 225/75 L (NOVO, Denmark) were used. In previous experiments different other amylolytic enzyme preparations were tested too but for citric acid fermentation the enzyme combination mentioned above proved to be the best *(8)*.

The conditions of starch hydrolysis were taken according to instructions of enzymes producer *(9)*.

For preparation of 500 g of starch hydrolysate the conditions were as follows:

A. Liquefaction:

starch B	140 g
enzyme (diluted to 1:10)	3 mL
$CaCl_2 \cdot 2H_2O$	500 mg
water	to 500 g

Liquefaction was carried out at 75 °C for 30 minutes using constant stirring 500 rpm at a pH value of 6.5.

B. Saccharification:

Saccharification was carried out immediately after liquefaction by adding 5 mL of a 1:10 diluted enzyme preparation. The saccharification conditions were: temperature = 55 °C, pH = 4.5, reaction time = 8 h, stirring = = 250 rpm.

The degree of hydrolysis expressed as dextrose equivalent (DE) ranged from 85 – 95.

All starch hydrolysates were used immediately after their preparation.

For comparative purposes a chemically defined substrate (CDS) was used *(10)*. To study the use of starch hydrolysates in citric acid fermentation, sucrose was replaced by starch hydrolysate. The composition of the fermentation substrate (in g/L) was as follows:

starch as hydrolysate	140
NH_4NO_3	2.5
KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.004

pH = 2.5 (adjusted with HCl)

In another series of experiments, prior to sterilization (120 °C, 20 min) the amounts from 0 to 300 mg/L of potassium hexacyanoferrate(II) were added to this substrate. All fermentation experiments were carried out in 500 mL buffled Erlenmeyer flasks containing 100 mL of sterilized substrate which were inoculated and put on a rotary shaker (100 rpm) at a temperature of 30 °C.

Analytical methods

The degree of hydrolysis was expressed as dextrose equivalent which was determined by the standard method *(11)*.

Amounts of citric acid were calculated as total citric acid by titration of the filtered fermentation broth with 0.1 M NaOH against phenolphtalein since the strain used produces almost exclusively citric acid *(12)*.

Reducing sugars were determined according to the Fehling method *(13)* and reported in % of residual sugars.

The growth of the fungus was followed microscopically by a Wild microscope and presented in microphotographs.

Results and discussion

Aspergillus niger is known for its amylolytic activity, i.e., the amyloglucosidase production. So it would be logical that it is able to produce citric acid on starch as sole carbon source. But the amylolytic activity of the productive strain used here proved to be too low for a reasonable production *(14)*. On the other hand, a substrate containing 14 % starch is of a solid, pudding like consistency, so it can not be used in a submerged fermentation but only in a surface culture. Furthermore, *A. niger* starts producing citric acid only if the osmotic pressure of the substrate is high enough at the very beginning of the process *(15)*. In order to fulfill these requirements of the fungus, starch has to be hydrolyzed before inoculation. For this purpose acid hydrolysis is not suitable as different infermentable carbohydrates such as dextrins as well as toxic by – products are produced *(4)*. Therefore, amylolytic enzymes were used.

Introductory citric acid fermentation experiments in substrates based on starch hydrolysates showed that inocula grown on beer wort agar slants did not produce satisfactory citric acid amounts compared to citric acid production in a chemically defined substrate with sucrose as the carbon source. As wheat starch B contains also proteins it could be a good nutritional medium. We adapted the strain to starch as the carbon source by using gradually higher addition of soluble starch for the inoculum cultivation as described in Materials and Methods. We substituted sucrose with soluble starch which lead to less sporulation on the slants. We selected slants with the best sporulated mycelia and used the spores for inoculation.

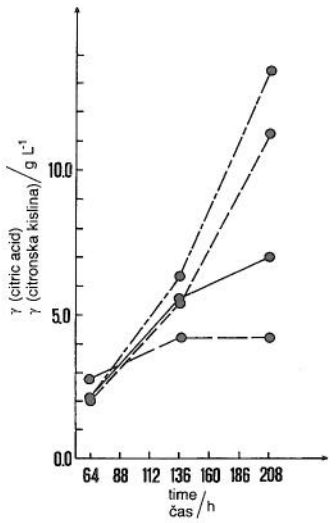

Fig. 1. Citric acid yields during fermentation using different inocula
Legend: Different substrates for inoculum preparation:
———— beer wort + 0.5 % soluble starch
— — — — beer wort + 1.0 % soluble starch
— · — · — beer wort + 1.5 % soluble starch
– – – – – – – 2 % soluble starch + inorganic salts

Slika 1. Dobitki citronske kisline med fermentacijo ob uporabi različnih vcepkov
Legenda: Različni substrati za pripravo vcepka
———— pivina + 0.5 % topnega škroba
— — — — pivina + 1.0 % topnega škroba
— · — · — pivina + 1.5 % topnega škroba
– – – – – – – 2 % topni škrob + anorganske soli

As shown in Fig. 1 citric acid yields were better when using inocula grown on soluble starch (1.5 or 2.0 %).

All further experiments were done using inocula cultivated on the solid substrate containing 2 % of soluble starch as sole carbon source.

It is known that the absence of heavy metal ions, especially of Mn^{2+} ions in the fermentation substrate is essential for good citric acid production. Maximal allowed Mn^{2+} concentration is 1 μg/L of substrate *(6)* while the average Mn^{2+} concentration in wheat starch B used in our work was 5.5 μg/g of dry matter. Thus, the concentration of Mn^{2+} in our substrate was 1200 μg/L.

As citric acid accumulation in starch hydrolysates was substantially lower compared to the chemically defined medium we tried to improve our results by adding different amounts of hexacyanoferrate to the fermentation medium. It was expected that the use of this additive could diminish the effect of metal ions present in untreated starch hydrolysates.

It was neccessary to find an optimal amount of hexacyanoferrate in order to shift the metabolism in the direction of higher citric acid accumulation and at the same time not to inhibit the fungal growth to a too high degree. After several experiments varying the addition of hexacyanoferrate we came to the results presented in Table 1.

It can be seen that by the use of hexacyanoferrate(II) it was possible to improve citric acid yields using starch

Table 1. Citric acid yields in g/L in starch hydrolysate substrate with different hexacyanoferrate(II) concentrations
Tabela 1. Dobitki citronske kisline na substratu iz škrobnega hidrolizata ob različnih koncentracijah heksacionoferata(II)

Time of cultivation / h Čas gojenja	Addition of hexacyanoferrate(II) / (mg L⁻¹) Dodatek heksacionoferata(II)					
	0	40	50	100	200	300
96	7.0	5.6	4.2	3.5	2.8	2.8
192	15.4	28.0	32.2	16.8	17.5	14.7
288	3.2	14.7	14.7	33.6	17.5	21.0

hydrolysates as carbon source in the fermentation medium. Optimal results were achieved after 192 hours of fermentation by adding 40 and 50 mg/L of hexacyanoferrate(II). At a higher hexacyanoferrate(II) concentration (100 mg/L) even better results could be obtained but after a longer fermentation period of 288 hours. The addition of 40 and 50 mg/L of hexacyanoferrate(II) doubles the citric acid content of the broth. After a longer fermentation time, e.g., 288 hours, the content of citric acid decreases in substrates with a lower concentration of hexacyanoferrate(II); but increases in those with higher concentrations. The phenomenon can be explained by inhibition of growth and metabolism of *Aspergillus niger* by higher concentrations of hexacyanoferrate(II) *(16,17)* and by early exhaustion of sugars in trials with lower hexacyanoferrate(II) concentration followed by dissimilation of citric acid *(18)*. For example, in tests without hexacyanoferrate(II) the fungus removed during the first 96 hours around 60 % sugar, till the 192nd between 90 and 95 % sugar, and till the 288th more than 99 % of initial sugar concentration.

Comparing the yields obtained using a sucrose containing substrate, i.e. 108 g L^{-1}, the results quoted in Table 1 are still low and the fermentation times rather long.

Comparing the fungal growth in the sucrose and in starch hydrolysates containing substrate, it was noticed that in the former case *Aspergillus* formed small dense pellets which is the optimal growth form for good citric acid yields (Fig. 2) whereas in the latter case the growth of the same strain was in the form of loose mycelial masses (Fig. 3).

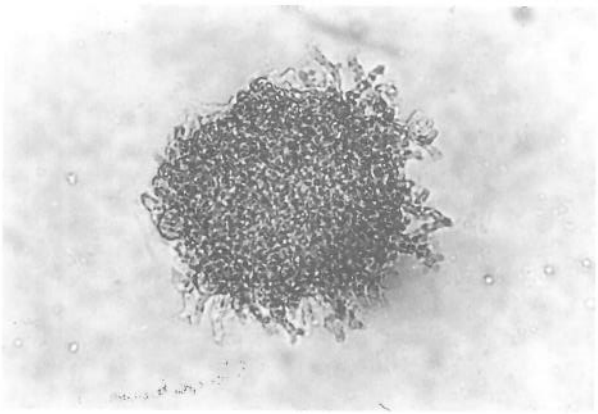

Fig. 2. Mycelial pellet, formed in CDS, after 136 h of incubation, 150 x
Slika 2. Micelijski pelet v CDS, po 136 h po cepljenju, 150 x

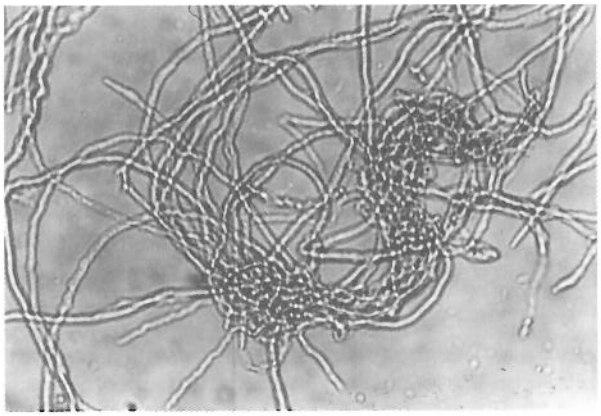

Fig. 3. Fungal mycelium in starch hydrolysate containing substrate, after 136 h of incubation, 150 x

Slika 3. Glivni micelij v substratu s škrobnim hidrolizatom, po 136 h po cepljenju, 150 x

Conculsions

Wheat starch B hydrolysates can be used as a possible alternative carbon source in citric acid fermentation.

Cultivation of the productive strain on gradually increased starch concentrations in the solid substrate for the inoculum preparation improved citric acid yields.

Using an inoculum mentioned above and adding appropriate amounts of hexacyanoferrate (II) to the fermentation substrate, additional improvements of citric acid yields could be achieved.

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