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ORIGINAL PAPER

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Ram horn peptone as a source of citric acid production by *Aspergillus niger*, with a process

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Abstract The present study deals with the production of citric acid from a ram horn peptone (RHP) by *Aspergillus niger* NRRL 330. A medium from RHP and a control medium (CM) were compared for citric acid production using *A. niger* in a batch culture. For this purpose, first, RHP was produced. Ram horns were hydrolyzed by treatment with acids (6 N H_2SO_4, 6 N HCl) and neutralizing solutions. The amounts of protein, nitrogen, ash, some minerals, total sugars, total lipids and amino acids of the RHP were determined. RHP was compared with peptones with a bacto-tryptone from casein and other peptones. The results from RHP were similar to those of standard peptones. The optimal concentration of RHP for the production of citric acid was found to be 4% (w/w). A medium prepared from 4% RHP was termed ram horn peptone medium (RHPM). In comparison with CM, the content of citric acid in RHPM broth (84 g/l) over 6 days was 35% higher than that in CM broth (62 g/l). These results show that citric acid can be produced efficiently by *A. niger* from ram horn.

Keywords *Aspergillus niger* · Agricultural waste · Citric acid · Protein hydrolysate · Ram horn

Introduction

Citric acid is used in the pharmaceutical, food and beverage industries as an acidifying and flavor-enhancing agent and is used in the production of synthetic medium, beet and cane molasses by surface and submerged fermentation using *Aspergillus niger*. In past years, considerable interest has been shown in using agricultural wastes for citric acid production. For this purpose, different agro-industrial residues, such as apple pomace, wheat straw, coffee husk, pineapple waste, cassava bagasse, banana, sugar beet cosset, kiwi fruit peel, etc., have been investigated as a substrate [1–4]. Citric acid production has always been a subject of interest for many workers. Various chemical, physical and biochemical techniques in the industrial processes for citric acid production have been investigated [5–8].

Peptones are defined as protein hydrolysates that are soluble in water and not coagulated by heat [9]. These products may have significant value for the fisheries industry, as they have somewhat higher market prices than the usual byproducts, such as fish silage and fishmeal. Growth substrate costs often make up the major part of the production cost of microbial cells and byproducts from the fermentation industry [10]. The nitrogen source is usually the most expensive component of bacterial growth substrates and at present is obtained from plants [11], dairy proteins (such as casein [12] or whey [13]) and slaughterhouse waste [14]. In contrast, peptones and fish hydrolysates are made either by acid hydrolysis or enzymatic digestion of proteins. Acid hydrolysis allows high yields; but this process results in high ash content in the final products as the neutralization step cannot be avoided [15].

Ram horn protein hydrolysate has been investigated only to a minor extent; and its use in industrial processes is limited. Ram horns make up a large amount of the waste products of the meat industry in Turkey. For example, slaughterhouses in Turkey directly discharge about 600 t/year. Increasing concern about the pollution that occurs from agricultural and industrial wastes has stimulated interest in converting waste materials into commercially valuable products, especially by single-cell protein production [16, 17].

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Furthermore, other fibrous proteins from feathers, nails and hair are available as waste. These waste products can be converted into biomass, protein concentrate or amino acids using proteases derived from certain microorganisms [18]. Ram horns consist of α-keratin, which is relatively rich in cysteine (up to 22%). In addition, they contain most of the other common amino acids [19–22].

When we started this research, we intended to compare peptone from ram horn with standard peptone. Experiments have been performed in this laboratory on ram horn protein hydrolysate, for the influence of ram horn hydrolysate on the crop yield of the mushroom *Agaricus bisporus* [23], for citric acid production [24, 25], for enhancement of glycerol production [26] and for peptone [14, 22], but this is the first time a peptone prepared from ram horn with our process has been tested for the production of citric acid. The hydrolytic processes for the above experiments involved several different methods; and this study contains another process for the hydrolysis of ram horn.

Since peptone is rather expensive, we examined the possibility of using peptone from ram horn as a complex nitrogen source. This paper deals with the different routes for the production of peptone from ram horn. It also defines a new medium (ram horn peptone medium; RHPM) based on ram horn peptone (RHP) and used in a fermentation for the production of citric acid by *Aspergillus niger*. RHPM citric acid production under laboratory conditions in batch fermentation is compared with a control medium (CM).

Materials and methods

Hydrolysis of ram horn

The chemicals used in this study were analytical grade and purchased from Oxoid (UK) and Difco (USA). Ram horns were obtained from the slaughterhouse at Erzurum, Turkey.

Hydrolysis processes were prepared by modifying the method of Kurbanoglu and Kurbanoglu [14]. For this purpose, horns were washed with deionized water and dried in an oven at 100°C to a constant weight. The dry horn was cut into smaller pieces and ground (Willey-Mill, Arthur, USA). The ground material was termed horn powder and a production scheme for RHP is shown in Fig. 1.

Organism and inoculum

A. niger NRRL 330 was supplied by Dr. C.P. Kurtzman (North University, Peoria, Ill., USA). The organism was maintained on potato dextrose agar (PDA) slants at 4°C which were renewed once at 1-month intervals. *A. niger* spores for inocula were produced on medium containing 50 ml PDA in a 250-ml Erlenmeyer flask, incubated at 28°C for 8 days. A spore suspension was prepared by adding 50 ml distilled water containing Tween-80 (2%) and was stored at 4°C for a maximum of 2 weeks. On average, it contained 10^4 spores/ml.

Media

CM contained (per liter): 140.0 g sucrose, 4.0 g NH_4Cl, 1.0 g KH_2PO_4, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.3 g $FeSO_4{\cdot}7H_2O$, 0.4 g $ZnSO_4{\cdot}7H_2O$, 0.15 g $MnSO_4{\cdot}2H_2O$, 0.4 g $CuSO_4{\cdot}5H_2O$ [5, 27]. RHP media contained (per liter): 140.0 g sucrose, 1.0 g KH_2PO_4 and RHP in various concentrations (1–8%).

Fermentation and growth measurement

A batch culture was established in a 2-l fermenter (M 880072/3, Biostat, Germany) with a working volume of 1 l. Spore suspension (5 ml) prepared as inoculum was used to inoculate a fermenter containing 500 ml of the medium being tested. The culture temperature was automatically maintained at 30°C. The initial pH of the culture medium was adjusted to 5.5 with 1 N H_2SO_4 and 1 N $Mg(OH)_2$. Agitation speed and aeration rate were kept at 200 rpm and 1.0 vol./min, respectively. Growth of the fungus was measured by dry cell weight (biomass) of filtered cells. Biomass was determined following filtration, drying the cell mass at 80°C overnight and weighing the resulting material. The supernatant was used for the determination of sugars and citric acid.

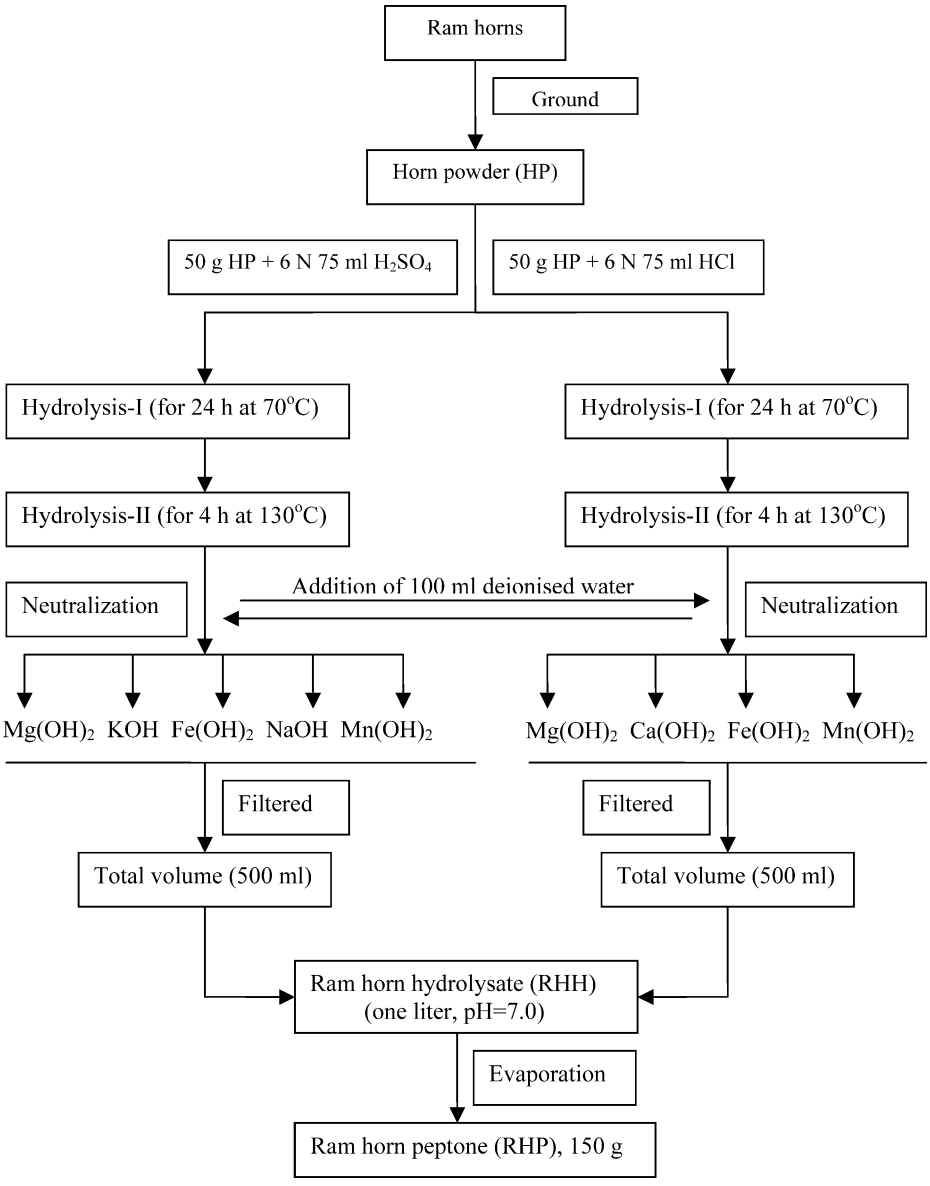
Analytical methods

Amino acid analysis was carried out after hydrolysis with 6 N HCl in a LC-5001 amino acid analyzer (Biotronic, Germany). Total sugar content, dry matter and ash analysis in the RHP were estimated by AOAC methods [28]. Total nitrogen was determined by a micro-Kjeldahl method [29]. Total lipids were estimated according to Folch et al. [30]. The elemental composition was measured using an atomic absorption spectrophotometer (model 360, Perkin–Elmer, Germany). Citric acid in the cultures was measured spectrophotometrically [31]. Residual sugar was determined by 3,5-dinitrosalycilic acid reaction [32].

Statistical analysis

The media were compared against each other. Experiments were replicated three times in a randomized block design. All data were analyzed using the general SAS linear models procedure. Differences between means were tested for significance ($P < 0.01$) by Duncan's multiple range tests [33].

Fig. 1 A production scheme for RHP



Results and discussion

A production scheme for RHP using our process is shown in Fig. 1. This process is somewhat different than those of our previous studies [14, 22–26]. Although the processes for the hydrolysis of ram horn in our previous studies utilized a single type of acid and neutralizing solution, this process uses two types of acid and many neutralizing solutions. Essentially, the objective of this process is to prevent the formation of both single-type salts and sulfates in the generated product. Kurbanoglu [26] offered clear advantages in using ram horn hydrolysate for enhancing glycerol production. Similarly, we find this process satisfies the experimental results of citric acid production from RHPM using *A. niger*. This has been considered a method of ram horn waste-treatment. However, there is interest in fermentation as a process for energy production due to the constantly increasing costs of both fossil fuels and chemical feedstocks. According to Kristinsson and Rasco [34], acid hydrolysis of proteins is used more commonly than hydrolysis under alkaline conditions. Total hydrolysis of fish protein substrate can be achieved in 18 h at 118°C in 6 N HCl. Following neutralization of the digest, the hydrolysate contains large amount of salt (NaCl), which can make the product unpalatable and interferes with functionality in food systems.

The main chemical composition of RHP and the amino acid composition of bacto-tryptone (a high-quality nitrogen source) are shown in Table 1. Notably, RHP contains the substances essential for microbial growth, including sources of nitrogen and minerals. The chemical composition of RHP is consistent with the findings obtained from investigations on the amino acid

Table 1 Amino acid composition of RHP produced from ram horn and bacto-tryptone, a high-quality nitrogen source used for the cultivation of microorganisms

Component (g/100 g)	RHP	Amino acid (g/100 g)	RHP	Bacto-tryptone[a]
Nitrogen	8.0	Aspartic acid	3.6	7.7
Protein	50	Threonine	1.7	4.5
Dry matter	98	Serine	2.6	6.1
Ash	47	Glutamic acid	7.3	24.2
Total sugars	2.6	Glycine	4.6	2.3
Total lipids	1.4	Alanine	2.8	3.5
K	1.0	Cysteine	2.5	0.0
Cu	0.2	Valine	2.9	6.7
Zn	0.6	Methionine	0.4	2.4
$MgSO_4$[b]	3.6	Isoleucine	1.5	4.4
K_2SO_4[b]	4.0	Leucine	3.6	8.3
$FeSO_4$[b]	4.5	Tyrosine	1.5	2.9
Na_2SO_4[b]	4.3	Phenylalanine	1.6	4.6
$MnSO_4$[b]	4.5	Histidine	0.7	2.5
$MgCl_2$[b]	3.6	Lysine	2.1	7.2
$CaCl_2$[b]	5.1	Arginine	4.2	3.4
$FeCl_2$[b]	4.8	Proline[c]	3.5	9.3
$MnCl_2$[b]	4.7	Tryptophan	Not determined	Not determined

[a]From Clausen et al. [39]
[b]From neutralization step based on a calculation
[c]Proline in RHP was determined according to Bates et al. [40]

composition of various fibrous proteins, such as nail [35–38], fish epidermis [19] and bovine hoof [20]. As the results in Table 1 are similar to results in our previous studies [14, 22–26], these results are not discussed. Generally, the amino acid content of bacto-tryptone is approximately twice that of RHP (Table 1). The reason for this is that the formation of salt from the neutralization step in the hydrolysis of horn results in a high dry matter content in RHP. Nevertheless, RHP has essential amino acids, indicating a high nutritional value both for food, as feed and as a nitrogen source in growth media for microorganisms. The cysteine content of RHP is lower than that of some other fibrous proteins [18–21, 35–38]. At this point, a satisfactory explanation cannot be offered. It is likely that cysteine is lost during the hydrolysis of ram horn. Another drawback of acid hydrolysis is the destruction of tryptophan, which is an essential amino acid. However, these drawbacks are not relevant to the use we propose.

First, we investigated the effects of RHP in various concentrations (1–8%) on residual sugar, biomass and citric acid concentrations under identical cultivation conditions. These results are given in Table 2. It is clear that citric acid accumulation, sugar utilization and biomass concentration increased as the concentration of RHP in the medium increased, up to 4%.Thereafter, the accumulation of citric acid, sugar utilization and biomass concentration decreased with increasing concentration. These rates are statistically significant ($P < 0.01$). As shown in Table 2, the highest biomass (24 g/l), citric acid (77 g/l) and the lowest residual sugar (13 g/l) for 5 days were obtained from 4% RHP. Thus, this study found that the optimal concentration for the production of citric acid was 4%. It was found that applications higher than 4% had an inhibitory effect. For example, the lowest biomass (3.5 g/l) and citric acid (3 g/l) concentrations were obtained from the application of 8% RHP. This inhibitory effect may be due to the high biochemical oxygen demand (BOD) of RHP and the presence of cell wall cations and some toxic materials. In addition, cell growth was inhibited by increasing concentrations of dissolved carbon dioxide and significantly affected by the concentration of dissolved oxygen [41]. These results suggested that RHP above 4% had a negative effect on the cell growth rate. Therefore, we used 4% RHP for further studies. This substrate was termed RHPM. In comparison with using CM for citric acid production, RHPM resulted in 87% higher citric acid production (RHPM 77 g/l; CM 41 g/l). This result suggested that RHP had a stimulating effect on citric acid accumulation.

A comparison of the main chemical composition of RHPM (prepared from 4% RHP) with that of CM is given in Table 3. Ammonium chloride is the main nitrogen source used for citric acid production under laboratory conditions [5, 27]. In this study, RHP was considered as a source of nitrogen and minerals in the fermentation medium, due to its amino acid and mineral content. It is clear that citric acid could be produced by the fungus

Table 2 Residual sugar, biomass and citric acid concentrations of *A. niger* after 5 days for 30°C, using different RHP concentrations and CM. Values given are means of three trials, each trial being examined in duplicate. Values in the same row without a common superscript differ significantly ($P < 0.01$). Values with the same letter are not significantly different

Medium	Biomass (g/l)	Residual sugar (g/l)	Citric acid (g/l)
1% RHP	6.5^{a}	105^{a}	20^{a}
2% RHP	11^{bc}	73^{b}	42^{b}
3% RHP	17.5^{d}	34^{c}	62^{c}
4% RHP	24^{e}	13^{d}	77^{d}
5% RHP	18^{d}	34^{c}	60^{c}
6% RHP	12^{bc}	74^{b}	40^{b}
7% RHP	7.4^{a}	108^{a}	18^{a}
8% RHP	3.5^{f}	132^{e}	3.0^{e}
CM	15^{g}	48^{f}	41^{b}

Table 3 Comparison of the main chemical composition of RHPM (prepared from 4% RHP) with that of CM

Component	RHPM (g/l)	CM (g/l) [5, 27]
Protein	20	–
Sucrose	140	140.0
NH_4Cl	–	4.0
K_2HPO_4	1.0	1.0
Cu	0.08	–
Zn	0.21	–
$MgSO_4$	1.44	0.25
K_2SO4	1.60	–
$FeSO_4$	1.8	0.3
Na_2SO_4	1.72	–
$MnSO_4$	1.8	0.15
$MgCl_2$	1.44	–
$CaCl_2$	2.04	–
$FeCl_2$	1.92	–
$MnCl_2$	1.88	–
$ZnSO_4$	–	0.4
$CuSO_4$	–	0.4
Total lipids	0.56	–
Other sugars	1.04	–

when RHP was employed as the source of nitrogen and mineral in the fermentation medium. In addition, sulfate ($MgSO_4$, $FeSO_4$, $MnSO_4$) did not need to be added to the fermentation medium as it was generated during neutralization of the ram horn hydrolysate (Fig. 1).

The effects of incubation time on citric acid concentration and residual sugar of the culture were investigated (Table 4). The use of RHPM in a fermentation had a significant effect on citric acid production and sugar consumption. The differences between the results of RHPM and CM are significant ($P < 0.01$). The maximum citric acid concentration in the RHPM was observed at 6 days. This value was 84 g/l; and the sugar consumption was about 98%. However, the citric acid in CM for the same incubation time was measured as 62 g/l and the sugar consumption for this medium was 94%. As a result, the performance of RHPM was 35% higher than that of CM. However, the maximum citric acid concentration in CM was observed at 7 days. This value was 66 g/l; and the sugar consumption was about 98%. Nevertheless, this value is lower than that of RHPM. After 7 days of incubation, the content of citric acid in RHPM is 27% higher than that in CM. CM gave its maximum citric acid after a long time (7 days), while RHPM gave its after a short time (6 days). Therefore, the new medium (RHPM) for citric acid production may be more economic. It resulted in improvements in both duration and fermentation rate. The levels of citric acid production and sugar consumption in RHPM were higher than those in CM for all incubation times. It is not surprising to find such differences between cultures in the rate of production of citric acid. As seen in Table 3, RHPM is rich in minerals and amino acids. These nutrients enhance growth and the higher citric acid production by RHPM in comparison with CM may be due to the presence of these components in the substrate. Wang et al. [42] reported that fermentation efficiencies decreased when fewer nutrients was present in the fermentation media. It was also reported that the activity of some enzymes decreases when amino acids rather than ammonium salts are used as nitrogen source [43]. In addition, it was reported that the presence of glutamic acid and aspartic acid stimulated citric acid production to the extent of 80% and 77%, respectively. Lysine stimulated citric acid production by 62%. However, serine did not influence the yield (50%) significantly, while the effect of cysteine was found to be detrimental [44]. As seen in Table 1, RHP is rich in glutamic acid and aspartic acid.

In conclusion, 150 g of RHP were recovered from 100 g of horn powder after hydrolysis (as in Fig. 1). We obtained 84 g of citric acid from 40 g RHP over 6 days. Thus, 600 t of HP could be used to produce 1,878 t of citric acid. RHP contains a high nutrient content for microbial growth. This process is technically feasible for the treatment of waste horn and the recovery of citric acid. Waste horns are disposed of by municipalities across Turkey, causing severe environmental problems due to the associated high organic pollutant BOD and microbial loads. Citric acid recovery from waste horn in Turkey can reduce this pollution problem. The production of citric acid from other fibrous proteins, such as feathers, nails and hair, should also be researched. The cost of RHP production was not considered in this study. It remains to be determined whether supplementation with RHP would be economic under commercial conditions. RHP has the potential to be a valuable supplement for use in citric acid production.

Table 4 Citric acid concentration (CAC) and residual sugar (RS) in grams per liter on RHPM and CM for various incubation times (4, 5, 6, 7 days). Values given are means ($\pm$SD) of three trials, each trial being examined in duplicate. Values in the same row without a common superscript differ significantly ($P < 0.01$). Values with the same letter are not significantly different

Medium	Incubation time (days)							
	4		5		6		7	
	CAC	RS	CAC	RS	CAC	RS	CAC	RS
CM	36 ± 0.4^a	63 ± 1.8^a	41 ± 0.4^a	48 ± 1.5^a	62 ± 0.4^a	9 ± 1.3^a	66	2
RHPM	53 ± 0.6^b	55 ± 1.4^b	77 ± 0.7^b	13 ± 1.9^b	84 ± 0.6^b	2 ± 1.2^b	–	–

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