Bioresource Technology 85 (2002) 125–129

BIORESOURCE TECHNOLOGY

Single-cell protein production from ram horn hydrolysate by bacteria

Esabı Basaran Kurbanoglu *, Omer Faruk Algur

Science and Letters Faculty, Department of Biology, Atatürk University, 25240 Erzurum, Turkey

Received 17 October 2001; received in revised form 1 March 2002; accepted 26 March 2002

Abstract

Ram horns obtained from the slaughterhouse of Erzurum, Turkey were hydrolyzed by treating with acid (6N–HCl) and ram horn hydrolysate (RHH) was obtained. The hydrolysate was used as substrate to grow *Bacillus cereus* NRRL B-3711, *Bacillus subtilis* NRRL NRS-744 and *Escherichia coli* in batch system at 30 °C; air 1.5 v/v/min; stirring 150 rpm. Both RHH and biomass samples were analyzed. The bacterial cells produced in this hydrolysate were found to be rich in total protein (66%, 68% and 71% for *E. coli*, *B. cereus* and *B. subtilis*, respectively). The chemical oxygen demand and biological oxygen demand were reduced significantly by the growth of bacteria. The protein produced contained all essential amino acids for ruminant feed.

Keywords: Single-cell protein; Horn; Fibrous proteins; Slaughterhouse waste; Bacteria

1. Introduction

Single-cell protein (SCP) refers to the dried cells of microorganisms. SCPs are used as protein sources in human foods or animal feeds. Many raw materials have been studied as substrates for the production of SCP. In many cases, these raw materials have been hydrolyzed by physical, chemical and enzymatic methods before use (Ferrer et al., 1996; Chanda and Chagrabarti, 1996; Nigam, 1998; Shojaosadati et al., 1999).

Ram horns make up a large amount of the waste products of the meat industry in Turkey. For example, the slaughterhouses in Turkey directly discharge about 600 tons a year. Increasing concern about pollution that occurs from agricultural and industrial wastes has stimulated interest in converting waste materials into commercially valuable products, especially SCP (Leman et al., 1990). Furthermore, other fibrous proteins from e.g. feather, nail and hair are available as waste. These waste products can be converted to biomass, protein concentrate or amino acids using proteases derived from certain microorganisms (Atalo and Gashe, 1993).

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 (Lehninger, 1975; Baden and Kubilus, 1983a,b; Dalev, 1990). The objective of this study was to evaluate ram horn hydrolysate (RHH) as a fermentation medium for the production of SCP by bacteria and thereby reutilize this abundant animal waste.

2. Methods

2.1. Ram horn hydrolysis

Ram horns were obtained from Slaughterhouse Erzurum, Turkey. The chemicals used in this study were analytical grade and purchased from Oxoid (UK) and Difco (USA). Horns were washed with deionized water and dried in an oven at 100 °C to constant weight. The dry horns were cut into smaller pieces and ground (Wileymill Arthur, USA). Obtained material was termed horn flour (HF). Thirty-five grams of the HF were impregnated with 50 ml of 6N–HCl. The resulting mixture was incubated at 80 °C for 24 h. At the end of this period, the mixture was incubated at 130 °C for 1 h after adding 100 ml deionized water. The solution was then cooled to room temperature and the pH adjusted to 7.0

* Corresponding author. Tel.: +90-442-2314342/2311943; fax: +90-442-2360948/2314342.
E-mail address: ekurbanoglu@yahoo.com (E.B. Kurbanoglu).

0960-8524/02/$ - see front matter
PII: S0960-8524(02)00094-9

with 10N–NaOH. It was then filtered twice through Whatman no. 1 filter paper. The volume was adjusted to 400 ml with deionized water. The final clear filtrate was termed RHH and stored at 4 °C until further analysis was completed. As a result of this procedure, 30 g of an initial 35-g HF was hydrolyzed. The RHH was enriched by the addition of 0.5% glucose. The effects of various concentrations (1–10%) of the RHH on the biomass yields of the bacteria were investigated.

2.2. Microorganisms and cultivations

Two bacterial strains, *Bacillus cereus* NRRL B-3711 and *Bacillus subtilis* NRRL NRS-744 were supplied by Dr. C.P. Kurtzman (1815 North University Street Peoria, Illinois 61604, USA). A third strain of *Escherichia coli* was isolated from wastewater of sugar factory in Erzurum, Turkey. These strains were grown on Nutrient agar (NA) slants (pH 7.2) at 30 °C for 24 h and then stored in a refrigerator at 4 °C by monthly transfers. Bacteria cells from stock cultures on NA slants were transferred then into 250-ml flasks containing 100 ml of Nutrient Broth medium which were previously sterilized at 121 °C for 15 min. The flasks were incubated for 24 h at 30 °C on a rotary shaker at 150 rpm. After growth, the bacterial samples were centrifuged at $5000 \times g$ for 20 min at 5 °C, and cell suspensions were prepared from harvested samples in a 100-ml sterile physiological salt solution. Inoculum size was about 5%; media pH was 7.2 before autoclaving. The incubation time required for maximum biomass generation was determined by trial and found to be 48 h for all the strains tested.

2.3. Batch culture and measurement of growth

A batch culture was established in a 2 l fermenter (BIOSTAT M 880072/3) with a working volume of 1.0 l. RHH was sterilized in an autoclave at 121 °C, 15 lb/in^2 for 20 min. The fermenter containing 1.0 l sterile RHH was inoculated with 5% (v/v) inoculum. Temperature (30 °C), agitation (150 rpm) and aeration rate (1.5 v/v/min) were kept constant, and foam controlled manually by addition of silicone. Growth of the bacteria was measured by dry cell weight of the harvested cells (biomass). The biomass was determined following centrifugation at $5000 \times g$ for 20 min, drying the cell mass at 80 °C overnight, and weighing resulting dry cell biomass.

2.4. Analytical methods

Chemical oxygen demand (COD) and biological oxygen demand (BOD) measurements were facilitated by diluting the sample 1:1000 and 1:1 through 1:1000, respectively, as required with redistilled water (Taras et al., 1971; Ballinger, 1979). Both RHH and biomass samples generated were analyzed. Amino acid analysis was carried out after hydrolysis with 6N–HCl in a Biotronic LC-5001 Amino Acid Analyser (Germany). Total sugar content, dry matter and ash analysis were estimated by AOAC methods (1980). Total nitrogen was determined by the microkjeldahl method (Byers, 1967). Total lipids were estimated according to Folch et al. (1963). The elemental composition was measured using an atomic absorption spectrophotometer (Perkin–Elmer 360 USA).

2.5. Statistical analysis

The experiments were replicated three times in a randomized block design. All data were analyzed using an SAS general linear model. Differences among means were tested for significance ($p < 0.05$) by Duncan's multiple range test (Ray, 1985).

3. Results and discussion

The main chemical composition of RHH is shown in Table 1. These data show RHH to be rich in both organic and inorganic materials. Notably, it contains the essential substances required in microbial media such as sources of carbon, nitrogen and minerals. In addition, RHH is rich in amino acids. The essential amino acids are present and among them arginine (4.66 mg/ml) was highest in concentration. However, of all the amino acids considered, glutamic acid (8.17 mg/ml) was the most abundant. The cysteine content was lower than that of some other fibrous proteins (Lehninger, 1975; Baden and Kubilus, 1983a,b; Dalev, 1990). At this point a satisfactory explanation cannot be offered. It is likely that the reduced content of cysteine is due to hydrolysis procedures of ram horn. The absence of tryptophan and

Table 1
The main chemical composition of RHH

Components (g/100-ml RHH)		Amino acids (mg/ml)	
Nitrogen	0.881	Aspartic acid	3.90
Protein	5.500	Threonine	2.00
Dry matter	8.800	Serine	2.87
Ash	1.98	Glutamic acid	8.17
Total sugar	0.500	Glycine	5.19
Total lipids	0.300	Alanine	3.19
Mg	0.160	Cysteine	0.21
Ca	0.164	Valine	2.56
Cu	0.017	Methionine	0.41
Mn	0.036	Isoleucine	1.63
Zn	0.064	Leucine	4.02
Fe	0.123	Tyrosine	1.61
K	0.113	Phenylalanine	1.67
		Histidine	0.72
		Lysine	2.21
		Arginine	4.66
		Proline	Not determined

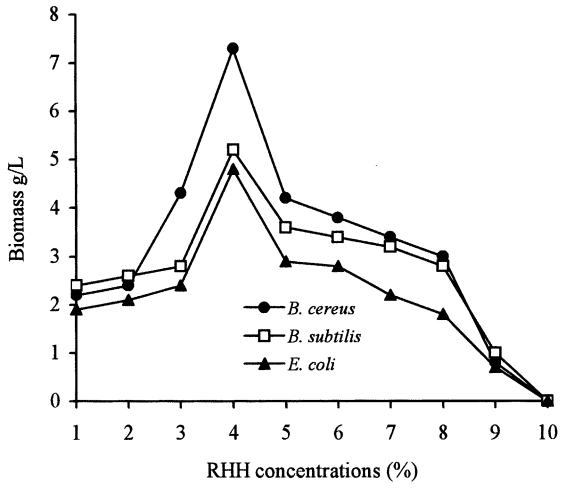

Fig. 1. The effects of different RHH concentrations on the biomass yields of test microorganisms.

proline was probably due to their degradation during the acid hydrolysis of proteins, because the hydrolysis procedure did not allow for the determination of some amino acids including proline and tryptophan (Lehninger, 1975). The chemical composition of RHH is in accordance with the findings obtained from previous investigations on the elemental and amino acid composition of the various fibrous proteins such as nail (Pruzanski and Arnon, 1966; Bank et al., 1981), fish epidermis (Baden and Kubilus, 1983a) and bovine hoofs (Baden and Kubilus, 1983b).

First of all, we have investigated the effects of RHH in various concentrations (1–10%) on bacterial biomass yields. The highest biomass yields for *B. subtilis*, *B. cereus* and *E. coli* were obtained from 4% RHH (Fig. 1). Thus in this study it was found that the optimal concentration for biomass growth was 4%. It was found that applications higher than 4% had an inhibitory effect. For example, the lowest biomass yields were obtained from the application of 9% RHH. Furthermore, no growth was observed from the application of 10% of RHH (Fig. 1). This inhibitory effect may be due to the high biochemical oxygen demand (BOD) load of RHH and presence of cell wall cations and some toxic materials. Similar effects have been observed with effluents with high loads of organic and inorganic materials (Kadioglu and Algur, 1992). Therefore, we continued the research with 4% RHH. The main chemical composition of substrate (1.0 l) is 40 ml of RHH and 5 g of glucose.

Table 2
Biomass and its partial composition obtained from RHH after 48-h growth

Biomass and its composition (g/100-g)	Types of bacteria[a]		
	B. cereus	*B. subtilis*	*E. coli*
Biomass yield (g/l)	7.3[a]	5.2[b]	4.8[c]
Crude protein ($N \times 6.25$)	68[a]	71[a]	66[a]
Lipids	8.1[a]	8.4[a]	8.1[a]
Ash	4.3[a]	4.1[a]	4.4[a]

[a] Each value represents the mean of three tests. Values with the same letter are not significant. Means in row without a common superscript differ ($p < 0.05$).

The biomass and resulting composition produced by bacteria are shown in Table 2. The differences in biomass yields among three bacteria were statistically significant, whereas the differences in partial compositions among three bacteria were not statistically significant ($p < 0.05$). The highest biomass yield (7.3 g/l) was obtained from *B. cereus*. Its biomass had a total protein content of 68%. The highest protein value (71%) of the biomass produced was observed for *B. subtilis*. The lowest biomass (4.8 g/l) and protein yield (66%) was obtained from *E. coli*. These three bacterial strains contained similar amounts of lipid, typically between 8% and 8.5% of dry weight. Similarly, ash ranged from 4.1% to 4.4%. A major limiting factor in the use of SCP as a food is its nucleic acid content. Bacterial cells are rich in total nucleic acids that unfortunately were not detected by the assays used in this study. Becker (1986) suggested that the high levels of nucleic acids in bacterial strains may limit their use as a food source for humans, who lack uricase (an enzyme converting uric acid, a toxic intermediate of nucleic acid catabolism, to the non-toxic allantoin). Therefore, high levels of nucleic acids in bacteria fed to ruminants should not have toxic effects. For this reason, we produced SCPs for use as ruminant feed.

The percentage reductions of COD and BOD, as well as efficient utilization of C and N sources are shown in Table 3. Maximum BOD and COD reductions for *B. cereus* were 98% and 63%, respectively. In addition, both sugar and nitrogen sources in RHH were consumed by bacteria effectively. The biomass produced had a total protein content of 68% (*B. cereus*), 71% (*B. subtilis*) and

Table 3
Utilization of sugar and nitrogen and reduction of COD and BOD of RHH by bacteria

Bacteria[a]	Utilization of sugar (%)	Utilization of nitrogen (%)	COD of RHH (mg/l)	Reduction (%)	BOD of RHH (mg/l)	Reduction (%)
B. cereus	97	58	30 400	63	21 050	98
B. subtilis	91	60	30 400	58	21 050	84
E. coli	92	59	30 400	51	21 050	79

[a] Each value represents the mean of three cultures.

Table 4
Comparison of the amino acid profile of the protein produced by bacteria with that of other proteins

Amino acids	As percentage of total protein					
	B. cereus[a]	*B. subtilis*[a]	*E. coli*[a]	FAO[b]	Soya bean[b]	Ruminants feed[c]
Non-essentials						
Alanine	7.4	9.0	7.0	–	–	–
Aspartic acid	10.6	10.6	8.8	–	–	–
Cystine	1.2	0.7	1.0	2.0	1.6	0.74
Glutamate	12.9	12.6	10.1	–	–	–
Glycine	5.9	6.6	6.1	–	–	2.43
Serine	4.4	4.7	5.4	–	–	–
Tyrosine	4.0	5.0	5.5	–	–	–
Essentials						
Arginine	7.8	7.9	8.8	–	–	–
Histidine	1.8	2.3	2.1	–	–	–
Isoleucine	4.4	4.6	5.1	4.2	4.9	2.57
Leucine	8.9	8.7	7.2	4.8	8.0	3.80
Lysine	5.7	4.5	4.9	4.2	6.6	3.20
Methionine	2.3	2.6	2.7	2.2	1.3	0.72
Phenylalanine	5.1	4.9	6.3	2.8	5.3	2.20
Proline	4.1	4.4	3.9	–	–	–
Threonine	5.4	5.2	6.2	2.8	4.3	1.97
Trytophan	–	–	–	1.4	1.4	0.60
Valine	6.0	6.1	6.3	4.2	5.0	2.70

[a] Values are means of duplicates.
[b] From Araujo and D'Souza (1986).
[c] From Lo and Moreau (1986).

66% (*E. coli*). Their respective amino acid compositions are shown in Table 4, together with the amino acid profiles of two animal feed protein standards. The amino acid profiles of the proteins produced by three bacteria were similar. All of the bacteria contained most of the amino acids essential for animal feed. Moreover, they have a superior profile when compared with Food and Agricultural Organization (FAO) and the soya bean meal. The products were also rich in sulfur-containing amino acids. For example, the three bacteria are rich in methionine, around 2.3–2.7%, which is comparatively higher than that of other proteins.

4. Conclusions

In short, the amount of HF utilized was 35 g, and the amount of HF generated by hydrolysis was 30 g. According to this result, 85.7% of horn can be utilized as a growth medium for SCP production by bacteria. In this work, we obtained 73 g of biomass from 35-g HF for *B. cereus*. In this case, 600 tons of HF can be converted to 1251 tons of SCP. Horns are a waste product of the meat industry. These horns are disposed by municipal sewers to landfills, causing severe environmental problems due to its high organic pollutant (BOD) and microbial loads. Thus, the SCP recovery from waste horns in Turkey may significantly reduce this pollution problem. In this research, SCP had not been taken into consideration as to how much production cost would be increased or decreased if this technique was used under commercial conditions.

Acknowledgements

This work was supported by the Department of Biology, Science and Letter Faculty, Atatürk University. The authors thank G. Biringen, researcher in The Scientific and Technical Research Council of Turkey (TUBITAK), for amino acid analysis.

References

AOAC, 1980. Official Methods of Analysis, 13th ed. Association of Official Agricultural Chemist, Washington, DC.

Araujo, A., D'Souza, J., 1986. Enzymatic saccharification of pretreated rice straw and biomass production. Biotechnol. Bioeng. 28, 15039.

Atalo, K., Gashe, B.A., 1993. Protease production by a thermophilus *Bacillus* species which degrades various kinds of fibrous proteins. Biotechnol. Lett. 15, 1151–1156.

Baden, H., Kubilus, J., 1983a. The fibrous protein of fish epidermis. J. Invest. Dermatol. 80, 36–38.

Baden, H., Kubilus, J., 1983b. Fibrous proteins of bovine hoof. J. Invest. Dermatol. 81, 220–224.

Ballinger, D.G., 1979. Methods for Chemical Analysis of Water and Waste. Environmental Monitoring and Support Laboratory, US Environmental Protection Agency, OH, USA.

Bank, H.L., Robson, J.B., Bigelow, J.B., Morrison, J., Spell, L.H., Kantor, R., 1981. Preparation of fingernails for trace element analysis. Clin. Chim. Acta 116, 179–190.

Becker, E.W., 1986. Nutritional properties of microalgae: Potential and constraints. In: Richmond, A. (Ed.), Handbook of Microalgal Mass Culture. CRC Press, Boca Raton, FL, pp. 339–420.

Byers, M., 1967. Practical Details for the Small Scale Extraction of Protein from Leaves. Rothamstead Experimental Station, Herts, UK, p. 5, line 260.

Chanda, S., Chagrabarti, S., 1996. Plant origin liquid waste: A resource for single-cell protein production by yeast. Bioresour. Technol. 57, 51–54.

Dalev, P., 1990. An enzyme-alkaline hydrolysis of feather keratin for obtaining a protein concentrate for fodder. Biotechnol. Lett. 12, 71–72.

Ferrer, J., Paez, G., Marmol, Z., Ramones, E., Garcia, H., Forster, C.F., 1996. Acid hydrolysis of shrimp-shell wastes and the production of single cell protein from the hydrolysate. Bioresour. Technol. 57 (1), 55–60.

Folch, J., Less, M., Sloane-Stanley, M., 1963. Isolation and purification of total lipids from tissues. Biol. Chem. 226, 497–509.

Kadioglu, A., Algur, O.F., 1992. Tests of media with vinasse for *Chlamydomonas reinhardii* for possible reduction in vinasse pollution. Bioresour. Technol. 42, 1–5.

Lehninger, A.L., 1975. Biochemistry, second ed. Worth Publishers, 444 Park Avenue South, New York, p. 1104.

Leman, J., Bednarski, W., Grewal, H.S., 1990. Single-cell protein: current status. Ind. J. Microbiol. 30, 13–28.

Lo, S.N., Moreau, J.R., 1986. Mixed culture microbial protein from waste sulfite pulping liquor II. Its production on pilot plant scale and use in animal feed. Can. J. Chem. Eng. 64, 639–646.

Nigam, J.N., 1998. Single cell protein from pineapple cannery effluent. World J. Microbiol. Biotechnol. 14 (5), 693–696.

Pruzanski, W., Arnon, R., 1966. Determination of cystine and other amino acids in the fingernails of members of various ethnic groups in Israel. J. Med. Sci. 2, 465–467.

Ray, A.A., 1985. SAS Users Guide: Statistic, 1982 ed. SAS Institute Statistical Analysis System, Cary, NC, p. 584.

Shojaosadati, S.A., Khalilzadeh, R., Jalilzadeh, A., Sanaei, H.R., 1999. Bioconversion of molasses stillage to protein as an economic treatment of this effluent. Resour. Conserv. Recycl. 27 (1–2), 125–138.

Taras, M.J., Greenberg, A.E., Hoak, R.D., Rand, M.C., 1971. Standard Methods for the Examination of Water and Wastewater, XII ed. American Public Health Association, Washington, DC.