BIOTECHNOLOGY

A METHOD FOR PROCESSING OF KERATIN-CONTAINING RAW MATERIAL USING A KERATINASE-PRODUCING MICROORGANISM STREPTOMYCES ORNATUS S 1220

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Abstract: The effect of substrate on mycelium growth, the optimal composition of the culture medium, and the optimal cultivation conditions for Streptomyces ornatus S 1220 have been investigated in the present work. The specific activity of keratinase has been monitored during cultivation and activity variation caused by addition of various salts to the cultivation medium has been analyzed. The results of the optimization study are reported and successful use of the culture studied in the present work in processes performed on an industrial scale is anticipated.

Key words: keratin, secondary raw materials, processing, keratinase-producing microorganism, enzyme, protein, cultivation, keratinase activity, bioconversion

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INTRODUCTION

The amount of secondary raw material obtained during slaughter and processing of poultry can be as high as 45% of the live weight of the birds. Most of this material is constituted by down and feathers, which have a high biological value, since they contain about 85% keratin.

Keratins are abundant proteins found in epithelial cells. They are major structural components of skin, nails, hair, feathers, and wool. Analysis of the amino acid composition of keratins shows that these proteins are a rich source of essential amino acids. However, the transformation of natural keratin into a digestible form is problematic, since keratins are fibrillar proteins and their mechanical stability is higher than that of all other materials of biological origin except chitin. The conventional techniques used for the processing of keratin-containing raw materials are neither efficient nor rational. The use of physical and chemical processing methods can result in formation of various toxic substances, as well as in loss of up to 75% of protein. Consequently, novel procedures for the processing and efficient use of the secondary raw material are needed.

Enzymatic methods for the processing of protein-containing raw materials, which became available due to the development of biotechnology, allow for the preservation of all essential amino acids. The use of ready-made enzyme preparations on an industrial scale can lead to a significant increase in costs and expenses, and therefore it is necessary to find solutions which enable the minimization of processing costs for the keratin-containing raw material. The use of live microorganism cultures in the processing of keratin-containing raw material allows for a reduction of the processing costs. The bioconversion method involves cultivation of the enzyme-producing strains on a substrate formed by the raw material to be processed. High efficiency of the subsequent decomposition of the substrate can be attained if this method is used. Selection of the optimal enzyme-producing microorganism and the optimal cultivation conditions is necessary in order to increase the rate and efficiency of bioconversion employing this method.

With the requirements concerning the strain and its functional efficiency taken into account, we chose the keratinase-producing strain Streptomyces ornatus S 1220. Simple composition and low cost of cultivation media, high levels of keratinase production, short cultivation time, and high enzyme yield [4] were the primary reasons for the choice of this strain.

The aim of the present work, formulated with the current problems taken into account, was to define the optimal composition of the cultivation medium providing for a high biomass yield of Streptomyces ornatus S 1220, to determine the most appropriate cultivation temperature and the period during which the rate of biomass accumulation is the highest, and to assess the effect of chemical additives on the specific enzyme activity. The tasks to be fulfilled in order to achieve the aim included analysis of the chemical composition of feathers, characterization of the effect of complex cultivation media on the yield of keratinase produced by the microorganism, analysis of environmental effects on the growth and productivity of the microorganism under investigation, and optimization of the cultivation parameters established.

MATERIALS AND METHODS

The concentration of cations in the feathers was determined using a Kapel'-105 capillary electrophoresis system, a VSL-200 laboratory balance, and an SM-50 centrifuge. Total nitrogen/protein concentration was determined using a Rapid N cube total nitrogen/protein analyzer and a VSL-200 laboratory balance. Protein content in the biomass was determined according to European standards using a RAPID N ELEMENTAR protein analyzer. The method is based on combustion of a pre-weighed portion of the substance under investigation. The combustion performed in the presence of oxygen at a high temperature (approximately 900°C) results in the formation of carbon dioxide, water, and nitrogen; the amount of the latter is measured by the device.

The nutrient media used in the present work included starch-based medium (GOST (State Standard) R 52060-2003), selective Czapek medium (TU (Technical Specifications) 9229-014-00419789-95), and meat-peptone agar (MPA, GOST (State Standard)-17206-96). The effect of chemicals present in the substrate on the increase of the specific keratinase activity was assessed using cultivation of Streptomyces ornatus S 1220 on nutrient broth supplemented with one of the following salts: NaCl, KH_2PO_4, or $CaCO_3$. Keratinase activity was assayed using a modification-based method: namely, 200 mg of ground feathers (rinsed with chloroform and water and air-dried) were mixed with 10 mL of 0.05 M borate buffer, pH 9.0, and 1 mL of culture filtrate, shaken vigorously and incubated at 37 ± 1°C for 3 hours in order for keratin hydrolysis to occur. Two control incubations were started simultaneously in order to assess the solubility of feathers in the buffer and the content of soluble protein in the culture medium. Residual non-cleaved protein was precipitated by trichloroacetic acid (TCA) and separated by filtration. The optical density at the wavelength of 340 nm was measured in the filtrate. The amount of cleaved protein ($\mu g/cm^3$) per 1 h of incubation with the culture medium was determined using a calibration curve constructed using serum albumin solutions.

RESULTS AND DISCUSSION

The total content of protein in the feathers was approximately 87.5% by mass, as determined in the experiments described above. The content of cations in the feathers was determined as well. The results are shown in Table 1.

Investigation of the composition of the feathers confirmed that feathers are a product of high biological value and showed that they contain cations essential for the growth of the streptomycete [4].

Temperature is among the most dramatic effects exerted on a living organism by the environment. A living organism adapts to specific temperature conditions. Temperature determines the growth rate of a microorganism and has an impact on all aspects of its physiological functioning. The growth rate of microorganisms can either decrease or increase as the temperature changes. Since the reported optimal temperature for mycelial growth is about 30°C [3], we cultivated the microorganisms at 30 ± 1 or 37 ± 1°C on one of the three culture media: starch agar, MPA, or Czapek medium. Inoculated plates were placed in a thermostat at temperatures indicated above.

Table 1. Mass concentration of cations in feather

Parameter	Hydrolyzate volume, mL	Cation concentration in the sample, mg/dm^3		Average value, mg/dm^3	Content of the cation in the sample, x±Δ, g/kg
		1	2		
Potassium	12.5	6.861	6.794	6.828	17.07±1.72
Sodium	12.5	16.888	16.675	16.782	41.96±4.22
Magnesium	12.5	1.795	1.775	1.785	4.46±0.45
Calcium	12.5	8.933	8.357	8.645	21.61±0.22

The most suitable medium and temperature for biomass expansion were established by cultivating Streptomyces ornatus S 1220 on media described above for 7 days and measuring the protein content in the biomass. The colonies grown on the plates were counted and the concentration of microorganisms per 1 g biomass was determined. The amount of biomass was maximal after 7 days of cultivation on all three culture media tested, but the rate of biomass accumulation was maximal during the first three days of cultivation and decreased significantly afterwards. The optimal cultivation temperature was 30 ± 1°C for all three culture media tested. The results are shown in Table 2.

Table 2. Biomass accumulation during the cultivation of Streptomyces ornatus S 1220 at different temperatures

Temperature, °C	Number of microorganisms, $\times 10^{-3}$ CFU/g			
	Day 1	Day 3	Day 5	Day 7
Starch agar				
30.00	146	345	360	375
37.00	16	35	50	52
MPA				
30.00	200	590	630	645
37.00	20	100	105	110
Czapek medium				
30.00	101	235	250	265
37.00	10	35	38	40

Table 2 shows that the keratinase-producing microorganism becomes less active as temperature increases and that biomass accumulation is the most intensive upon cultivation on meat-peptone agar.

In a subsequent experiment, the total protein content was monitored during 7 days. The experiment was carried out at 30°C, since this temperature provided for the most intensive biomass growth. The total protein content was calculated by multiplying the total nitrogen content by a coefficient which is equal 6.25 for living organisms. The results are shown in Table 3.

A relatively intensive protein accumulation was detected in the culture grown on MPA at 30°C, with the protein content amounting to about 70% on the seventh day of cultivation. Therefore, meat-peptone agar was chosen for further studies.

Table 3. Protein accumulation in the biomass during cultivation of Streptomyces ornatus S 1220 at the optimal temperature (30ºC)

Protein content in the mycelium, mass %			
day			
1	3	5	7
Starch agar			
2.04	18	24	26
Nutrient agar			
5.6	59	68	70
Czapek medium			
5.9	22	26	30

After the optimal conditions for the cultivation of Streptomyces ornatus S 1220 were determined, further experiments aimed at the optimization of parameters affecting keratinase activity were performed.

The rate of an enzymatic reaction is affected by multiple factors. Selection of nutrient media components providing for an increase of the specific activity of keratinase is necessary to delineate the best conditions for the conversion of keratin-containing raw materials into amino acids.

The effect of salts (NaCl, KH_2PO_4, and $CaCO_3$) added to the cultivation medium on the specific activity of keratinase was monitored during 7 days. The concentration of the salts was in accordance with the earlier studies and equaled 0.25% for NaCl, 0.1% for KH_2PO_4, and 0.3% for $CaCO_3$ [1, 2]. After cultivation, the culture liquid was separated from the mycelium by centrifugation (3500–4000 rpm), and the supernatant was filtered. Keratinase activity of the filtrate was assayed [5]. The values of specific keratinase activity were registered on the first, third, fifth, and seventh days of cultivation. The results are shown in Table 4.

Table 4. Changes of the specific activity of keratinase during cultivation of Streptomyces ornatus S 1220

Specific activity of keratinase, U/mg protein			
day			
1	3	5	7
NaCl			
3.4	11.2	12.7	13.2
KH_2PO_4			
2.6	11.0	13.2	14.7
$CaCO_3$			
2.5	11.7	12.8	13.5

As shown in Table 4, the specific activity of keratinase increased throughout the 7 days of cultivation in media supplemented with the salts named above, but the highest value of the specific activity of keratinase was achieved after 7 days of cultivation in the presence of KH_2PO_4. The activity increased significantly in all samples after three days of cultivation, however, the activity increase was most pronounced in the culture supplemented with calcium carbonate (11.7 U/mg).

Besides, titratable and active acidity and specific keratinase activity were monitored during seven days of cultivation at 30°C in a medium not supplemented with any salts. The results are shown in Table 5. No changes in active acidity were detected; it remained at the level of 6.5 units during the 7 days of cultivation. Titratable acidity decreased from 18 to 16°T during the time period from day 3 to day 7, this being indicative of a decrease of the total amount of acids in the culture medium towards the end of the incubation. At the same time, the specific activity of keratinase increased significantly during the first three days and continued to increase slightly until the end of cultivation. This implies a connection between the increase of keratinase concentration and the high rate of biomass accumulation during the first three days of cultivation.

Table 5. Changes of pH, T, and keratinase activity during the cultivation of Streptomyces ornatus S 1220 on MPB at 30°C

Parameter	Day			
	1	3	5	7
Titratable acidity, °T	18	18	17	16
Active acidity, pH units	6.5	6.5	6.5	6.5
Specific keratinase activity, U/mg protein	2.6	11.0	11.9	12.8

CONCLUSION

The studies performed showed that the optimal duration of the cultivation of Streptomyces ornatus S 1220 is three days; the microorganism must be cultivated at 30°C on MPB supplemented with $CaCO_3$, since addition of the salt results in an increase of keratinase activity. The optimal duration of streptomycete cultivation is three days, since the rate of biomass accumulation is the highest during this time. Moreover, the activity of keratinase in the culture medium attains a value sufficient for efficient conversion of keratin-containing raw material after three days of cultivation.

In our opinion, implementation of the parameters defined in the present study will allow for efficient use of the Streptomyces ornatus S 1220 strain and increased yield of non-essential and essential amino acids during the processing of keratin derived from feathers. The use of this microbiological procedure for the processing of keratin-containing raw materials will allow for a decrease of the amount of waste in poultry-processing industry and provide a source of amino acids for feedstock, food, and pharmaceutical industry.

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