Brazilian Journal of Microbiology (2001) 32:6-9
ISSN 1517-8382

PROTEASE OBTENTION USING *BACILLUS SUBTILIS* 3411 AND AMARANTH SEED MEAL MEDIUM AT DIFFERENT AERATION RATES

Maria Delia Pastor*; Graciela Susana Lorda; Antonio Balatti

IMIQA (Instituto de Investigación y Desarrollo en Microbiología y Química Aplicada), Facultad de Ciencias Exactas y Naturales, UNLPam, Santa Rosa, La Pampa, Argentina.

Submitted: September 23, 1999; Returned to authors for corrections: February 04, 2000; Approved: March 07, 2001

ABSTRACT

The influence of the addition of *Amaranthus cruenthus* seed meal to the medium, as nutrient and growth factor, on protease production by *Bacillus subtilis* 3411 was studied. Tests were carried out in a rotary shaker and in mechanically stirred fermenters. The influence of aeration was also evaluated. The addition of amaranth in a concentration of 20 g/L resulted in 400% increase in protease production. Aeration up to 750 r.p.m. and 1 L/L.min had a favorable effect.

Key words: Proteases, *Bacillus subtilis*, amaranth, fermentation

INTRODUCTION

The bacterial proteases have found wide scale industrial application. Industries in which proteases are used include the pharmaceutical industry, the leather industry, the manufacture of protein hydrolizates, the food industry and the waste processing industry. The aim of this work was to improve the production of alcaline protease by *Bacillus subtilis* 3411. Using a recommended medium (1, 8, 9), the effect of amaranth seed meal at different aeration rates on the enzymatic protease levels achieved in rotary shakers and stirred fermenter was studied. Amaranth seed meal is a pseudocereal which contains a good aminoacid distribution and a high level content of several vitamins, (2, 3, 4, 7, 11). Addition of amaranth seed meal as growth factor in the culture medium can be an important procedure for optimization of the process.

Finally, using a balanced amaranth seed meal medium, the effect of aeration conditions on the protease formation was evaluated.

MATERIALS AND METHODS

Microorganism

A strain of *Bacillus subtilis* NRRL 3411 kept in the medium Nº 1 (Table 1) as spores in peat was used. The peat, 200 mesh, was adjusted to a 12% humidity content. Five gram portions were put into tubes and sterilized at 121ºC for 3 hours. Once the sterilization process was over, the mixture was impregnated with 3 mL spore suspension obtained in medium Nº 1 (Table 1). Tubes were hermetically sealed by either a threaded cap or a plastic film cover upon cotton caps. Tubes were stored at 5ºC.

Media

Culture media are shown in Table 1. The medium used in process, Nº 3, was modified with the addition of *Amaranthus cruenthus* seed meal, 200 mesh. This material was used in different concentrations (g/L): 10; 20; 40; 60, 80.

Inocula

Each flask was inoculated with a peat-kept spore suspension in 5 mL sterilized distilled water, previously exposed to 10 minute, 100ºC thermal shock. (5, 13, 15).

Operating conditions

Inocula were developed in 250 mL Erlenmeyer flasks containing 50 mL of culture medium. Processes were carried out in 500 mL Erlenmeyer flasks containing 100 mL of culture medium incubated for 72 hours in a rotary shaker (250 r.p.m. and eccentricity 2.5 cm). In all these experiments culture media and containers were sterilized at 121ºC for 20 minutes. Assays

* Corresponding author. Mailing address: IMIQA (Instituto de Investigación y Desarrollo en Microbiología y Química Aplicada), Facultad de Ciencias Exactas y Naturales, UNLPam, Av. Uruguay 151, 6.300, Santa Rosa, La Pampa, Argentina. Telephone: (+54) 2954-436787. Fax: (+54) 2954-432679

in fermenter were carried out at different stirring conditions, namely: 350; 750 and 1000 r.p.m., the aeration rate was 1 L/L. Min. Fermentation studies were performed in a 5 liter New Brunswick stirred fermenter using 3 L of culture medium. A connection to monitors allowed the following measurements and control measures to be obtained, namely: pH, temperature and stirring, dissolved oxygen partial pressure, and foam control by means of an automatic addition process of an antifoam silicone agent through a peristaltic pump.

Amaranthus cruenthus seed meal was used, previously milled to 200 mesh.

Cell growth

Microbial growth was determined by optical density units (650 nm) and by dry weight. A 10 mL sample was taken and centrifuged at 5000 g for 20 minutes. Precipitate was washed with distilled water twice. Precipitate was then resuspended in water and dried at 100ºC until constant weight.

Determination of alcaline protease activity

To determine the alcaline protease activity of culture media, ANSON modified method was used (8). ANSON modified enzyme unit, uAPAM, is defined as that quantity of enzyme which produces soluble fragments in tricloroacetic acid 0.2 M equivalent to 0.5 µg tyrosine at 37ºC in 10 minutes.

Consumption of lactose

To determine lactose concentration in the culture medium, Miller's spectrophotometric method, which measures reducing sugar, was used (10).

Determining cell oxygen demand and Q_{02} respiratory coefficient

The oxygen demand was measured in a Warburg respirometer at 28ºC (14).

Oxygen absorption rate

The oxygen absorption rate (OAR) was measured by the sulphite method (10).

Dissolved Oxygen

The dissolved oxygen was measured with a sterilizable silver-lead galvanic probe.

RESULTS

For tests carried out in rotary shaker, cell oxygen demand was in the order of 240 mL O_2/L.h for dry weight concentrations of 8.4 g/L and oxygen dissolution values were in the order of 500 mL O_2/L.h

Table 1. Composition of culture media.

Composition (g/L)	Sporulation Nº 1	Inocula Nº 2	Process Nº 3
Lactose	-	10.00	20.00
Glucose	1.00	-	-
Yeast extract	5.00	5.00	6.00
Peptone from meat (Britania Nº 1)	5.00	5.00	20.00
KH_2PO4	-	1.00	1.50
K_2HPO4	-	1.00	1.50
$CaCl_2.2H_2O$	-	-	0.06
$MnCl_2.4H_2O$	-	-	0.01
NaCl	-	-	1.50
$MgSO_4.7H_2O$	-	-	0.15
EDTA	-	-	0.56
Na_2SO_4	-	-	1.50
Tween 80	-	-	5.00
Agar	15.00	-	-
pH	6.9	6.9	6.9

Table 2. Influence of different concentrations of amaranth seed meal on proteases production, as determined in experiments performed in erlenmeyers on a rotary shaker at 250 r.p.m. and eccentricity 2.5 cm, for 72 hours.

Measured parameter	Amaranth seed meal concentration (g/L)					
(72 hours)	Control	10	20	40	60	80
Optical density units	18.18	5.1	8.7	13.7	14.3	12.0
pH	6.2	7.1	6.9	7.1	7.2	6.9
Lactose comsuption (g/L)	16.8	18	17.87	17.8	18.11	17.95
Dry weight (g/L)	8.0	8.1	8.4	9.3	9.6	8.8
Yield (g/g)	0.44	0.45	0.47	0.52	0.53	0.49
Specific cell growth rate (1/h)	0.029	0.029	0.030	0.031	0.030	0.030
Enzymatic activity (uAPAM/mL)	1065.0	2480.0	3950.0	1980.0	1960.0	1700.0
Specific enzymatic production (uAPAM/g)	133125.0	306172.5	470238.1	212903.0	204166.7	193181.8
Productivity (uAPAM/L h)	14791.7	34444.4	54861.1	27500.0	27222.2	23611.1

Table 2 shows results obtained in tests carried out in stirred erlenmeyers. In these experiments the influence of adding different concentrations of amaranth seed meal: 10; 20; 40; 60 and 80 g/L to a medium Nº 3, as shown in Table 1, which is used in turn as control, was considered.

It was observed that highest values of protease were obtained when a concentration of 20 g/L was added to the medium. Higher concentrations of amaranth seed meal showed lower enzyme activity, specific production and productivity, whereas biomass concentration, yields and specific growth rate were similar.

On the basis of results obtained in rotary shaker stirred erlenmeyers flasks, using the process medium with the addition of 20 g/L amaranth seed meal, new experiments were carried out in a stirred fermenter. The influence of aeration over process productivity was studied, using different stirring conditions and an air rate of 1 L/L min (Table 3). Oxygen dissolution rates used were similar and higher than those determined by rotary shaker, 529 mL O_2/L.h. Those values were for 350 r.p.m. = 562.43 mL O_2/L.h; for 750 r.p.m. = 2691 mL O_2/L.h and for 1000 r.p.m. = 5011 mL O_2/L.h. Fig. 1 show results obtained in these series of experiments. Table 3 also shows that the highest value of enzyme production was obtained at 750 r.p.m. with a protease level in the order of 11600 uAPAM/mL of culture, a specific growth rate of 0.029 1/h, a yield of 0.43 grams of biomass for lactose gram consumed, a biomass of 7.8 g/L of culture, specific production of 1487179.5 uAPAM for biomass gram, productivity of 161111.1 uAPAM for litre of fermented culture for hour. In all processes the evolution of pH values was almost near pH 7.

Table 3. Effect of aeration condition on the protease production in stirred fermentor.

Measured parameter	Agitation (r.p.m.)		
(72 hours)	350	750	1000
Lactose consumption (g/L)	17.9	18.0	17.7
PH	7.1	7.0	7.4
Optical density units	14.0	13.0	13.0
Dry weight (g/L)	7.0	7.8	8.0
Specific cell growth rate (1/h)	0.027	0.029	0.029
Yield (g/g)	0.39	0.43	0.45
Enzymatic activity (uAPAM/mL)	3950.0	11600.0	10000.0
Specific enzymatic production (uAPAM/g)	564285.7	1487179.5	1250000.0
Productivity (uAPAM/L.h)	54861.1	161111.1	138888.9

DISCUSSION

The considerable increase (400%) in production of proteases in rolary shakers, should be assigned to the important contribution of amino acids and vitamins from amaranth seed meal (3).

The protease synthesized by *Bacillus subtilis* NRRL 3411 has a molecular weight of 27400 d and 275 amino acid residues, with a large proportion of aspartic acid, serine, glicine, alanine and valine (8). With reference to the amaranth composition, the components of the material used in our studies in g/100 g

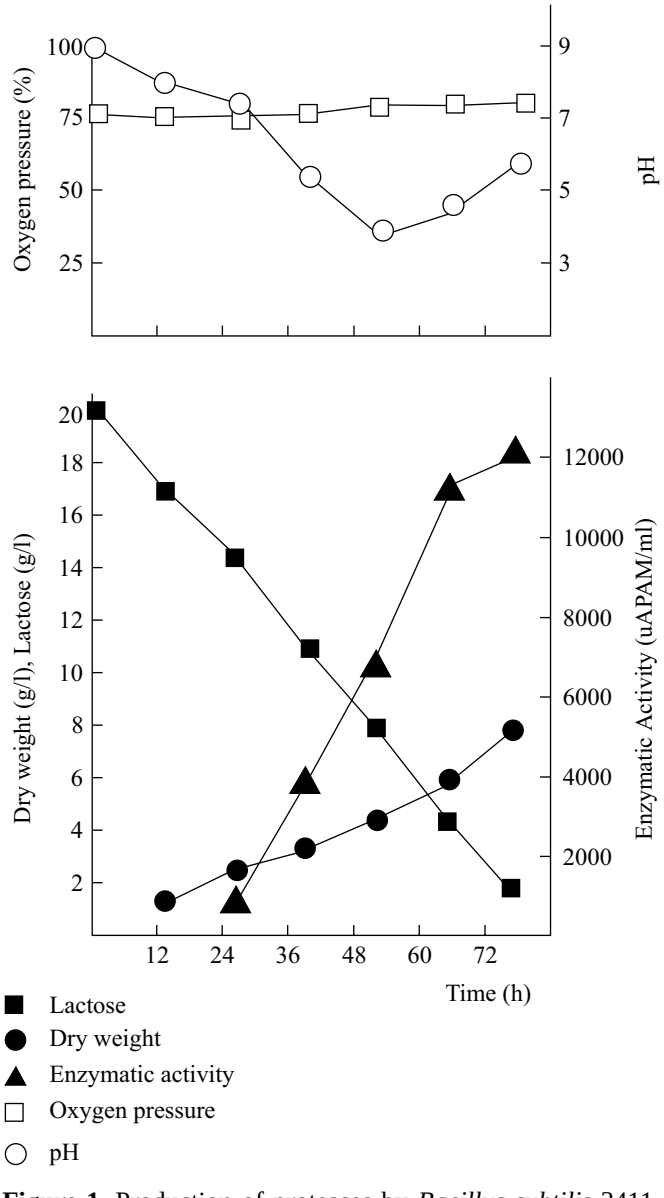

Figure 1. Production of proteases by *Bacillus subtilis* 3411, growing on seed meal amaranth (20 g/L medium), in mechanically stirred fermentor (750 r.p.m. and 1 L/L.min).

of protein were: aspartic acid, 2.8; serine, 2.3; glycine, 2.7; alanine, 1.3 and valine, 1.5. Thus, in the presence of amaranth seed meal, there will not be a limitation for these amino acids as might occur in media obtained from traditional sources. Besides, the content of vitamins (mg/100 dry weight) was thiamine, 0.07; riboflavin, 0.19; folic acid, 43.80 and ascorbic acid, 4.90 (4).

Adding amaranth seed meal in concentrations higher than 20 g/L did not produce an increase in enzyme production. This could be due either to the increase in the viscosity of the fermenting medium, limiting transference phenomena both of oxygen and metabolites, or to the fact that under such conditions, an inhibitory effect might occur due to an increase in amino acid concentration provided by amaranth.

If the results obtained in fermenters for similar oxygen dissolution rates are compared to those obtained in rotary shakers, 350 r.p.m., they will correspond, as expected, to equal rates of specific production. The highest enzyme values, obtained at 750 r.p.m., may be assigned to a greater oxygen availability in the culture medium where oxygen pressure was never below 30% of the saturation value as can be seen in Fig. 1.

However, higher agitation rates, 1000 r.p.m., increased the system's oxygen pressure but did not bring about production increase due, probably, at a high agitation rate, enzyme structure would be altered (12).

ACKNOWLEDGEMENTS

We thank the Facultad de Ciencias Exactas y Naturales, UNLPam, for finantial support.

RESUMO

Obtenção de protease usando *Bacillus subtilis* 3411 e meio com farinha de semente de *Amaranthus* em diferentes condições de aeração

Neste trabalho estudou-se a produção de proteases a partir de *Bacillus subtilis* 3411 cultivado em um meio no qual adicionou-se farinha de semente de *Amaranthus cruentus* como fonte de nutrientes e fatores de crescimento. As experiências foram realizadas em Erlenmeyers com agitador em fermentador de laboratório. Além disso, considerou-se a influência da aeração sobre a produção enzimática. A adição de amaranto em uma concentração de 20 g/L produziu um aumento de 400% no nível de proteases. A aeração das culturas teve um efeito favorável até valores de 750 r.p.m. empregando um fluxo de ar de 1 L/L. min.

Palavras-chave: Proteases, *Bacillus subtilis*, amaranto, fermentação

REFERENCES

1. Balatti, A.P. *Producción de enzimas*. Primer Simposio Interamericano sobre Biotecnología de Enzimas, México, 1984, p.89-107.
2. Becker, R. Amaranth Oil: Composition, processing and nutritional qualities. *In*: O. Paredes-López (ed). *Amaranth Biology, Chemistry and Thechnology.* U.S.A., 1994, p.133-140.
3. Bressani, R. *Grain amaranth. Its chemical composition and nutritive value.* Proceeding of the Fourth National Amaranth Simposium. Perspectives on Production, Processing and Marketing, Minnesota, 1990, p.23-25.
4. Bressani, R. Composition and Nutritional Properties of Amaranth. *In:* O. Paredes-López (ed). *Amaranth. Biology, Chemistry and Technology.* U.S.A, 1994, p.185-206.
5. Chiasson, P.; Zamenhof, L.P. Studies of induction mutations by heat in spores of *Bacillus subtilis. J. Microbiol.*, Vol. 12, 1966.
6. Cooper, C.M.; Ferston, G.; Miller, S.A. Gas Liquid Contactor. *Ind. Eng. Chem*., 36 : 504-509, 1944.
7. Covas, G. Revista Amarantos. Novedades e Informaciones. Estación Experimental Agropecuaria Anguil, INTA, Facultad de Agronomía, UNLPam, Argentina, 19: 1-12, 1995.
8. Kelly, C.; Fogarty, W. Microbial alcaline enzimes. *Proc. Biochem.*, 7: 3-9, 1976.
9. Massucco, A.E.; Mazza, L.A.; Balatti, A.P. *Obtención de preparados enzimáticos destinados a la depilación de pieles. Biotecnología de Enzimas.* Primer Simposio Interamericano sobre Biotecnología de Enzimas, México, 1981, p.271-277.
10. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31 : 426-428, 1959.
11. Ravindran, V.; Hood, R.L.; Gill, R.J.; Kneale, C.R.; Bryden, W.L. Nutritional evaluation of grain amaranth (*Amaranthus hypochondriacus*) in broiler diets. *A.F. Sci. Technol.*, 63 : 323-331, 1996.
12. Roychoudhury S.; Parulekar S.J.; Weigand W.A. Cell Growth and a-Amylase Production Characteristics of *Bacillus amyloliquefaciens*. *Biotechnol. Bioeng.*, 33 : 197-206, 1988.
13. Ruzal, S.; Sanchez Rivas, J. Physiological and genetic characterization of the osmotic stress response in *Bacillus subtilis. Can. J. Microbiol.*, 40 : 140-144, 1993.
14. Umbreit, W.W.; Burris R.H.; Sauffer J.S. The Warburg constant volume respirometer. *In:* Burgues Publishing Co. *Manometric and Biochemical Techniques*, U.S.A., 1972, p.1-17.
15. Zamenhof, S. Effects of heating dry bacteria and spores on their phenotype and genotype. *Proc. Natl. Acad. Sci.* 46 : 101-105, 1960.