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Formulation of culture media using fish scale bioconversion

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Short Communication

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- Jéssica Ferreira Mafra: Substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data.
- Paulo Sérgio Pedroso Costa Júnior: Substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data.
- Thiago Alves Santos de Oliveira: Substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data.
- Elizabeth Amélia Alves Duarte: Substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. Involvement in drafting the manuscript or revising it critically for important intellectual content.
- Aline Simões da Rocha Bispo: Involvement in drafting the manuscript or revising it critically for important intellectual content.
- Norma Suely Evangelista-Barreto: Substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. Involvement in drafting the manuscript or revising it critically for important intellectual content.

ABSTRACT

The biodegradation of fish scale residues is an alternative method to convert crude proteins into bioproducts of industrial interest. In view of this panorama, the objective of this study was to evaluate the use of tilapia scales as a supplement for the growth of *Bacillus subtilis.* For this, the work was divided into two stages. In stage 1, degradation assays with *Bacillus* isolates (B1, B2, and B3) were carried out in minimal mineral medium (MMM), supplemented with 1 g of fish scales, to which 1 mL of inoculum (10^8 UFC mL^{-1}) was added. The culture was incubated for six days at 125 rpm and 37 °C, and the pH was measured at the end of the degradation. In stage 2, two scale-based culture media (broths C1 and C2) were formulated. The efficiency of the formulated media was determined by the specific growth rate μ (h^{-1}) and the doubling time t_d (h) of *B. subtilis* (B1). Isolate B1 presented with the highest degradation yield from the treatment with the crushed scales and with a higher increase in the pH value (7.8) in the medium. In broth C1, the *B. subtilis* microorganism (B1) had a higher specific growth rate (1.9363 h^{-1}) and a shorter replication time (21.5 min). Therefore, different species of *Bacillus* were able to degrade the tilapia scales, and this product was found to be efficient in the formulation of culture media. This work demonstrates the application of novel technologies for the use of agroindustrial residues from the fish industry, resulting in a financial gain for the industry itself.

Keywords: agroindustrial residues, *Bacillus subtilis*, generation time, microbial growth

INTRODUCTION

With an increase in fish consumption around the world, a large amount of waste is generated every year. Therefore, it is necessary to utilize these waste products effectively in order to reduce the environmental impact they cause. It is estimated that the fish processing industry generates 18-–30 million tons of fish residue annually, out of which 4% are fish scales.[1]

Fish scales are primarily composed of organic and inorganic matter. The organic fraction consists of collagen and keratin proteins, and the inorganic fraction consists of hydroxyapatite (calcium phosphate), which is used by the industrial sector in cosmetics and biomedical products.[2,3,4]

The difficulty in the treatment of the scale residues can be attributed to the rigidity of keratin, which is a fibrous protein present in scales. Therefore, a viable, promising, and sustainable alternative that can be used to explore the protein potential of fish scales is its bioconversion through enzymatic processes.[5] Several microorganisms, such as fungi, bacteria, and actinobacteria, produce keratinases for keratin degradation, as do most of the ram-positive bacteria that belong to the genus *Bacillus.*[6,7] These enzymes are known to possess potential biotechnological applications for the bioconversion of residues.[8] However, certain factors, such as the microbial species, composition of the culture medium, type of fermentation, pH, temperature, etc., are considered to be important for an efficient degradation process and enzymatic production.[9]

The production of keratinase can generally be performed in relatively inexpensive growth media that contain keratinous substrates as the major sources of carbon and nitrogen; this makes the production of this enzyme highly favorable from an economic point of view.[8] Therefore, it can be inferred that the bioconversion of fish scales is performed by keratinolytic microorganisms. Although certain studies have previously reported the use of residues as substrates for microorganisms, ours is the first report on the use of fish scales by *Bacillus* species. The results of this study demonstrate that the use of bioproducts in culture media is rather promising when taking into account the microbial growth under the tested conditions.

In research laboratories, culture media are commonly used to supplement the nutritional requirements of microbial cells. However, the expenses related to these media can greatly burden the laboratory activities. Therefore, the interest in inexpensive formulations, aimed at the use of residues, has grown over

time, in order to achieve environmental preservation and a favorable cost-benefit ratio. Against this panorama, the objective of the present study was to analyze the efficiency of the biodegradation of Nile tilapia (*Oreochromis niloticus*) scales for their use as a substrate for the growth of a *Bacillus* species, with an aim for substitution in the formulations of high-cost culture media.

MATERIAL AND METHODS

First, *Bacillus* sp. was isolated from fish scales. For this, 5 g of scale residues was added to 5 mL of a 1.5% saline solution, and after 1 h at 80 ºC, the solution was cooled to activate the spores and destroy the vegetative cells. After a serial dilution to 10^{-3}, a 20 µL aliquot of each dilution was seeded on nutrient agar with 1.5% NaCl. The plates were incubated at 37 °C for 48 h.[10]

Subsequently, the fish scale isolates, as well as the strains of *Bacillus* spp. from the UFRB (University Federal of the Recôncavo of Bahia) collection that were capable of growth on a crushed scale agar medium [($g.L^{-1}$): 1 tryptone, 0.5 yeast extract, 1 NaCl, 5 scales, and 15 agar; pH 6.5; 35 °C/48 h)] and milk agar (37 °C/48 h), were selected. Growth in these media demonstrated a capacity to produce proteolytic enzymes. In the milk agar, the strains that presented with halos around the colonies were considered positive for enzyme production.[11]

The results obtained in each experiment were submitted to the analysis of variance (ANOVA), followed by Tukey's test ($p \leq 0.05$), using SISVAR 5.6 program.[12]

Three strains of *Bacillus* from a previous isolation were subjected to degradation assays: B1, B2, and B3. The identification of all isolates was performed at the genus level through morphological and biochemical criteria[13], according to methodologies proposed in the Bacteriological Analytical Manual (BAM) described by Silva et al.[14], and the isolate with the highest degradation was identified at the molecular level.[15]

The isolates (B1, B2, and B3) were cultured via submerged fermentation in whole scales broth (WSB) and crushed scales broth (CSB). The broth media contained ($g.L^{-1}$): 0.05 $MgSO_4.7H_2O$, 0.005 $ZnSO_4.7H_2O$, 0.015 $FeSO_4.7H_2O$, 0.025 $CaCl_2$, 10 glucose, 10 yeast extract, and 17 whole or crushed scales. The initial pH of the broths was adjusted to 6.5 before sterilization at 121 °C for 20 min. Erlenmeyer flasks (250 mL) with 60 mL of WSB or CSB were inoculated with 1 mL of a bacterial suspension solution (10^8 $CFU.mL^{-1}$) and incubated at 37 °C and 125 rpm for six days. At the end of the sixth day of degradation, the pH values of the media were measured. Negative (no microorganism) and positive (no scale) controls were incubated under the same conditions.

The broths were filtered through filter paper that was previously dried at 60 °C for 24 h, and the filter papers containing the remaining scale residues were dried under the same conditions and weighed to determine the percent degradation, as proposed by Reginato and Teixeira.[16]

To determine the growth kinetics, the bacteria were then subjected to culture in formulated and commercial media. The culture media formulations were as follows: broth C1 (5 g of crushed scales, 5 g glucose, 1.5 g yeast extract, 5 g sodium chloride, and 1 L distilled water, autoclaved at 121 °C for 15 min), and broth C2 (hydrolysate from the previous degradation of 1 g of crushed scales by the selected microorganism, sterilized by filtration).

The concentration of the scales was based on the composition of the commercial medium (nutrient broth) (HiMedia, Mumbai, India), aiming at the replacement of the animal peptone with the fish scales.

The performance of the microorganism in the formulated and commercial media was evaluated by the construction of growth curves. Erlenmeyer flasks (500 mL) containing 180 mL of nutrient broth [0.5% peptone, 0.5% sodium chloride, 0.15% meat extract, and 0.15% yeast extract] or 180 mL of the formulated media were inoculated with 1 mL of the standardized

microorganism (10^8 CFU.mL^{-1}) and incubated at 35 ± 0.5 °C at 125 rpm. At intervals of 0, 1, 2, 3, 6, 12, 24, and 48 h, a 100 µL culture aliquot was diluted to 10^{-5} in an Eppendorf tube containing 900 µL of an 0.85% saline solution. Then, 20 µL of each dilution was inoculated in triplicate on a Petri dish containing Plate Count Agar (PCA) medium (HiMedia, Mumbai, India) and incubated at 35 ± 0.5 °C for 24–48 h.

The growth kinetics of the selected microorganism were used to verify the efficiency of the culture medium (broth C1 or C2) compared to the control medium (nutrient broth). The specific growth rate µ (h^{-1}) and the time of duplication t_d (h) of the bacteria were obtained from the growth curves, which were generated by measuring the number of viable cells (CFU.mL^{-1}) as a function of time (h).

RESULTS AND DISCUSSION

The degradation yield obtained from the whole scales broth (WSB) for the isolates of the genus *Bacillus* ranged from 15% to 21%. Whereas, the degradation yield from the crushed scales broth (CSB) was higher, ranging from 22% to 26%. Isolate B3 demonstrated the highest yield for whole scale degradation, and it differed statistically from the others. When ground scales were used, isolate B1 demonstrated the highest yield at 26% (Table 1).

Table 1. Yield and pH for the degradation of the tilapia scales by the bacteria of the genus *Bacillus*.

Isolates	Whole scale degradation (%)	Degradation of crushed scale (%)	pH with whole scale	pH with crushed scale
B1	16bB	26aA	7.0bA	7.8cA
B2	15bB	22aA	4.6aA	4.6aA
B3	21aA	22aA	4.6aA	6.1bB
C.V.%	2.47	1.22	2.11	2.98

The values followed by the same lowercase letter in each column and the same capital letter in a row do not differ statistically, according to Tukey's test ($p \leq 0.05$). The experimental yield data were adjusted in cos x^{-1}.

The best yield was obtained for the degradation of the crushed scales, which can be attributed to the greater ease with which previously crushed substrates can be hydrolyzed, due to the greater area of contact with the substrate. The biodegradation observed can be explained by the production of extracellular enzymes, such as collagenases, keratinases, and other proteases, which hydrolyze the peptide bonds of the substrate and use them as a carbon source.[17]

A parameter that strongly influences the production of enzymes in microorganisms and the transport of several components be through the cell membrane is the pH.[18] The hydrolysis of keratin is generally accompanied by an increase in the pH of the culture medium. The tendency for an increased pH in the culture medium is a result of the production of ammonia from amino acid deamination and peptides from keratin degradation. Moreover, the release of calcium phosphate from the scales during the degradation process may also contribute to the elevation of the pH.[4]

Table 2. Maximum growth of *Bacillus subtilis* in the formulated media and nutrient broth.

Treatments	Cell growth CFU/mL
Broth C1	1.2×10^9 b
Broth C2	1.3×10^9 b
Commercial nutrient Broth	4.9×10^8 a
C.V.%	1.51

The values followed by the same letter do not differ statistically by Tukey's test ($p \leq 0.05$). The experimental data were adjusted for log.

The use of fish scales in the formulation of a culture medium is possible, considering the efficiency of the development of *Bacillus subtilis* [(Ehrenberg, 1835) Cohn, 1872] in the media that contained the scales compared to the commercial culture medium (nutrient broth). Cell growth of 10^9 CFU.mL^{-1} was achieved for both culture media, and this differed statistically from the growth observed in the nutrient broth (Table 2); this may have occurred because glucose was only present in the formulated media.

The compositions of broth C1 and the nutrient broth differed by only two ingredients. In broth C1, peptone was replaced with the crushed scales, and the meat extract was replaced with glucose, as an alternative, to provide an initial carbon source for the microorganism, in order to support microbial degradation.

Figure 1 depicts the growth curves obtained for the *B. subtilis* cultures in the three culture media tested (C1, C2, and nutrient broth).

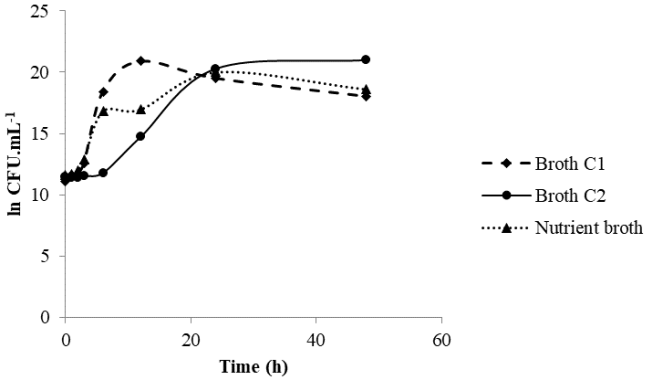

Figure 1. Growth curves for the cultivation of *Bacillus subtilis* in the fish scale broth (C1 broth), hydrolyzed protein fish broth (C2 broth), and commercial nutrient broth.

In broth C2, the duration of the exponential growth phase was longer than that observed for broth C1. However, broth C2 exhibited the characteristic curve for bacterial growth with well-defined phases (Fig. 1). The growth in the nutrient broth was diauxic, demonstrating two lag phases: the first one with a duration of 2 h and the second one with a duration of 6 h. This may be attributed to the sequential use of two or more carbon and energy sources in the medium, as a consequence of the mechanism of catabolic repression.[19]

Table 3 shows the results obtained in relation to the microbial growth of *B. subtilis*, which include the growth rate, the analysis of variance, doubling time, and the duration of the lag phase in each of the media tested.

In broth C1, *B. subtilis* exhibited a higher growth rate and a shorter doubling time, when compared to C2 and the nutrient broth. Among the media tested, broth C2 was the medium in which *B. subtilis* duplicated most slowly. After the observed diauxic growth in the nutrient broth, the growth rate of the microorganism decreased, as shown in Figure 1.

Table 3. The growth kinetics of *B. subtilis* in the three different culture media.

Broth	μ (h^{-1})	t_d (min)	Duration of the phase lag (h)
Broth C1	1.9363	21.5	2
Broth C2	0.4718	88.2	3
Nutrient broth	1.3040	32.0	2

μ: Specific growth rate, t_d: generation time

The growth of *B. subtilis* in the fish stock broth (broth C1) caused an abrupt decrease in the instantaneous growth rate, after the exponential phase had transitioned to the stationary phase. Meanwhile, in the hydrolyzed protein broth (C2 broth), this transition was gradual; this difference may be explained by the depletion of glucose that occurred in broth C1.

The variation observed between the growth profiles for the C1 and C2 broths could be related to the composition of the media. In the C2 broth, the source of carbon and energy was basically the hydrolyzed keratin and collagen compounds; whereas, in broth C1, in addition to glucose, the yeast extract, NaCl, and 0.5% of crushed scales were available. The media formulated with scales fulfilled the nutritional requirements of *B. subtilis*, as the scales are composed of organic molecules that can act as a source of carbon, nitrogen, and energy.

It is possible that the short lag phase observed for broth C1 and the nutrient broth may be due to the greater availability of nutrients. The lag phase is the preparation period for proliferation, which may be short or several hours long, depending on the composition of the original medium, composition of the new medium, type and age of the strain, genetics of the microorganism, the inoculum, physical factors, such

as the pH, agitation, temperature, etc., and even on the presence of substances produced during the metabolism of the microorganism, such as organic acids.[20]

In view of this panorama, the utilization of fish scale residues by microorganisms and their subsequent application in the industrial sector appears rather promising. However, it is necessary to perform tests capable of determining the proteolytic activity and to optimize the degradation process, as well as to evaluate the viability of the formulations.

CONCLUSIONS

This study showed that a new *Bacillus subtilis* (B1), isolated from fish scales, showed the capacity to degrade recalcitrant substrates in submerged cultures. Therefore, the same could be used in the bioconversion of tilapia scales by means of fermentation, in order to reduce the amount of industrial solid waste, which makes it possible to use them in the formulation of culture media to replace the commercial means available on the market. Additional studies are needed to quantify the activity of the enzymes involved in the degradation process, as well as to optimize the production process for future biotechnological applications.

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