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"Gheorghe Asachi" Technical University of Iasi, Romania

SARDINELLE BY-PRODUCT HYDROLYZATES AS NITROGEN SOURCE FOR MICROBIAL GROWTH AND PROTEASE PRODUCTION

Nabil Souissi[1]*, Mourad Jridi[2], Rabeb Ben Slama[2], Faouzi Ben Rebah[3], Moncef Nasri[2]

[1]*Laboratory of Biodiversity and Marine Biotechnology, National Institute of Marine Sciences and Technologies. Tunisia*
[2]*Laboratory of Enzyme Engineering and Microbiology, National Engineering school of Sfax. Tunisia*
[3]*King Khalid University, Community College at Khamis Mushait, PO Box 3926 - 61961, Saudi Arabia*

Abstract

Tunisian fish-processing industries generates large amounts of by-products. Their disposal is an increasing environmental and health problem. For an economic utilization, these by-products may be used in producing higher value-added products such as protein hydrolyzates. In this study, protein hydrolyzates with different degrees of hydrolysis (12%, 20% and 26%) were prepared from heads and viscera of *Sardinella aurita* using crude protease of *Bacillus licheniformis* NH1. The obtained hydrolyzates containing 60% to 63% of proteins are tested as nitrogen or carbon sources for microbial growth and protease production. Results indicated higher level of protease activity for *Bacillus cereus* SV1 (5100 U/mL), *Bacillus amyloliquefaciens* An6 (2000 U/mL) and *Pseudomonas aeruginosa* MN7 (4200 U/mL). Interestingly, best protease production depends on the degree of hydrolysis (DH), justifying the use of different hydrolysis conditions. Sardinelle by-product hydrolyzates were also found to be good substrates for *Escherichia coli* and *Saccharomyces cerevisiae* growth. This approach can reduce environmental problems associated with fish waste disposal and, simultaneously, lower the cost of microbial growth and enzyme production.

Key words: biomass, fish by-product, protease, proteolytic strains, sardinelle protein hydrolyzate

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1. Introduction

Protected areas have many benefits such as Due to its high protein content, low-cost fish species and fish by-products represent a potential source of industrial peptones. Peptones from fish species have appeared in media catalogues only in the last decade. Particularly, fish protein hydrolyzates have been reported as substrate for bacterial growth Dufossé et al. (1997). In Tunisia, Sardinelle (*Sardinella aurita*) is abundant exceeding 15,000 tons in 2011 (Shatz, 2000). Generally, this fish is exploited as raw material for canning industries, but large part of its capture is still underutilized. For better management of Sardinelle catches it is a challenge to utilize the valuable protein fraction from the by-product to produce higher value-added products such as fish protein hydrolyzate or peptones. Organic nitrogen substrates, are widely used in many biological and biotechnological applications, such as microbial biomass production (Singh et al., 1995; Yousuf et al., 2017), and metabolite biosynthesis including enzymes (Agrawal et al., 2017; Haltrich et al., 1994; Rapp, 1995). At present, peptones are obtained from casein, soy protein, gelatin, meat (Boonmee, 2012; Reissbrodt et al., 1995) and various agro-industrial residues (Moreira et al., 2012). The use of fish protein hydrolyzates for maintaining the growth of different microorganisms has received a great amount of attention (Clausen et al., 1985; De la Broise et al., 1998; Dufossé et al., 2001; Gildberg et al., 1989), but only limited number of studies reports the application of this substrate to metabolite production (Coello et al., 2000). Ghorbel et al. (2005) have reported the

* Author to whom all correspondence should be addressed: e-mail: nabil.souissi@gmail.com; Phone: +21674497117; Fax: +21674497989

production of lipase by *Rhyzopus oryzae* in medium containing glucose and fish protein hydrolyzate as nitrogen source. Souissi et al. (2008) and Souissi et al. (2009) reported the use of cuttlefish and Sardinelle by-products for the production of proteases and lipases respectively. More recently, a significant improvement of the alkaline protease production by *Bacillus mojavensis* A21 was obtained using Sardinella peptone (Haddar et al., 2010). In this work, we investigate the preparation of protein hydrolyzates from Sardinelle (*S. aurita*) by-products (heads and viscera) using a simple and low-cost process, and explore their utilization as nitrogen and/or carbon sources for biomass and protease production.

2. Materials and method

2.1. Fish sampling

Sardinelle (*S. aurita*) was obtained from the fish market of Sfax City, Tunisia. After washing with distilled water, heads and viscera were separated and used immediately for the preparation of protein hydrolyzates.

2.2. Microorganisms

Microorganisms used in this work, their origin, preculture and culture media, and growth conditions are summarized in Table 1. All microorganisms except *E. coli* and *S. cerevisiae* where isolated in the Laboratory of Enzyme Engineering and Microbiology, Sfax-Tunisia.

2.3. Preparation of sardinelle by-products protein hydrolyzates

Crude protease of *B. licheniformis* NH1 (El-Hadj Ali et al., 2007) was used for protein hydrolyzates preparation. The scheme for the production of protein hydrolyzates from Sardinelle heads and viscera is given in Fig. 1.

Heads and viscera (500 g) were first minced then cooked in an equal amount of distilled water and homogenized in a Moulinex blender for about 2 min. The pH of the mixture was adjusted to the optimum activity value for the hydrolysis enzyme. In order to have different hydrolysis degree, the enzyme was added to the reaction to give an enzyme/substrate ratio of 0.087 U/mg, 0.69 U/mg and 0.87 U/mg (units of enzyme by weight of Sardinelle's heads and viscera). The pH of the mixture was maintained constant during the enzymatic reaction by continuous addition of 4 N NaOH solution to the reaction mixture. After that, reaction was stopped by heating the solution at 80 °C during 20 min to inactivate the enzyme. The fish hydrolyzate was centrifuged at 5000 x g for 20 min to separate insoluble and soluble fractions. Then, to remove lipids, the mixture was centrifuged at 3000 x g for 20 min at 4 °C, the low temperature makes lipids solid and then facilitates their elimination. Finally, the soluble phase was dried in a ventilated oven at 80 °C for 2 hours. Samples were stored as hydrolyzed Sardinelle protein powder.

2.4. Determination of the degree of hydrolysis (DH)

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds by weight unit (h_{tot}), in each case, was calculated from the amount of base consumed as described by Adler-Nissen (1986).

2.5. Cultivation and media

Inocula were routinely grown in Luria-Bertani (LB) broth medium (g L^{-1}): casein peptone, 10.0 ; yeast extract (YE), 5.0 ; NaCl, 5.0 (Miller, 1972). Media were autoclaved at 120 °C for 20 min. Control media used for protease production are presented in Table 1.

Table 1. Microorganism used in this work and their specification

Microorganism	***Origin***	***Culture media***	***Conditions***	
			Preculture	***Culture****
Escherichia coli	Commercial MosCell	LB, pH = 70	14 h, 37°C, 200 rpm	24 h, 37°C, 200 rpm
Saccharomyces cerevisiae	Provided by « Centre de Biotechnologie de Sfax-Tunisia »	Glucose, 15; Casein Peptone, 4; KH_2PO_4, 2; $CaCl_2$, 013; $FeSO_4,7H_2O$, 001; $MgSO_4,7H_2O$, 3; pH = 72	22 h, 30°C, 150 rpm	72 h, 30°C, 150 rpm
Bacillus cereus SV1	Changing oil station for fishing boats	Starch 5; YE 2; $CaCl_2$ 1; KH_2PO_4 0,1; K_2HPO_4 0,1; pH = 80	22 h, 37°C, 200 rpm	48 h, 37°C, 200 rpm
Vibrio metschnikovii J1	Soap industry wastewater	Casein 10; YE 5; NaCl 5; pH = 80	18 h, 37°C, 200 rpm	18 h, 37°C, 200 rpm
Bacillus licheniformis NH1	Activated sludge reactor	Gruel, 10; YE, 2; KH_2PO_4, 0,5; K_2HPO_4, 0,5; ammonium sulfate, 2; $CaCl_2$, 1; $MgSO_4$, 10; pH = 80	18h, 37°C, 200 rpm	24 h, 37°C, 200 rpm
Bacillus amyloliquefaciens An6	detergent industry floor	Gruel, 30; YE, 6; KH_2PO_4, 0,1; K_2HPO_4, 0,1; $CaCl_2$, 1; pH = 80	18 h, 37°C, 200 rpm	24h, 37°C, 200 rpm
Pseudomonas aeruginosa MN7	tannery wastewater	Casein peptone, 10; $(NH_4)_2SO_4$, 1; YE, 3; KH_2PO_4, 0,5; K_2HPO_4, 0,5; $MgSO_4,7H_2O$, 0,1; $MnSO_4, H_2O$, 0,01; Glucose, 2; pH = 80	18 h, 37°C, 200 rpm	24h, 37°C, 200 rpm

*YT: Yeast Extract; MJTP: Mirabilis Jalapa Tuber Powder. * Conditions of growth to obtain the highest cell count*

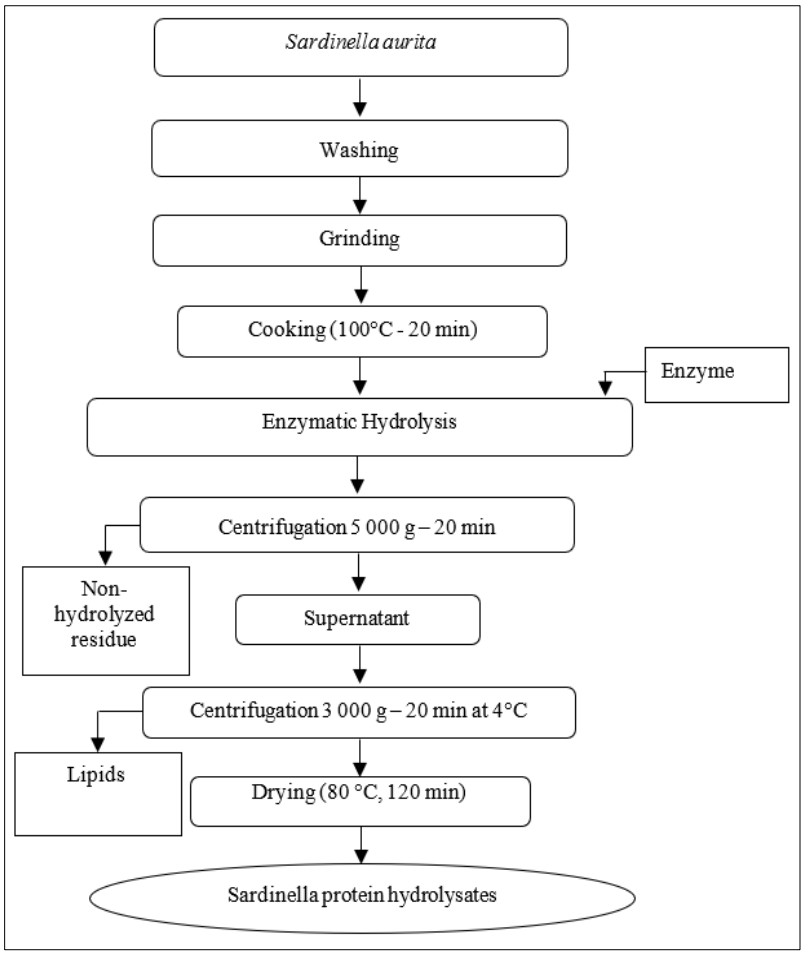


Fig. 1. A flow chart for the preparation of Sardinelle protein hydrolyzate (SPH)

Casein peptone was replaced by sardinelle protein hydrolyzates in the case of *E. coli*, *S. cerevisiae and P. aeruginosa* MN7, while in the case of *B. cereus* SV1, and *B. amyloliquefaciens* An6, yeast extract was replaced by fish protein hydrolyzates. This substitution was calculated keeping the same nitrogen/carbon ratio in all compared media. Cultivations were performed on a rotatory shaker, in 250 mL Erlenmeyer flasks with a working volume of 25 mL.

The cultures were centrifuged (8000 g, 15 min) and the cell-free supernatants were used to estimate the proteolytic activity. Cells growth was determined by measuring the optical density (OD) at 600 nm. All experiments were carried out in duplicate and repeated at least twice.

2.6. Chemical analysis

The moisture and ash content were determined according to the AOAC standard methods 930.15 and 942.05, respectively (A.O.A.C., 1995). Total nitrogen content was determined by using the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by the factor of 6.25. Lipids were determined gravimetrically after Soxhlet extraction of dried samples with hexane. All measurements were performed in triplicate.

2.7. Assay of proteolytic activity

Protease activity was measured by the method of Kembhavi et al. (1993) using casein as a substrate. All experiments were carried out in triplicate and repeated at least twice.

2.8. Statistical analysis

Statistical analyses were performed with STATGRAPHICS Centurion XV Ver. 15.2.05 (StatPoint, Inc.) using ANOVA analysis. Differences were considered significant at $P<0.05$.

3. Results and discussion

3.1. Sardinelle protein hydrolyzate preparation using B. licheniformis NH1 crude protease

The preparation of Sardinelle protein hydrolyzates was performed according to the process shown in Fig. 1. The cooking step is intended not only to inactivate the endogenous enzymes of the Sardinelle, but also to prepare the proteins of fish for the hydrolysis step. The hydrolysis is realized using the enzyme preparation of *B. licheniformis* NH1 as hydrolytic enzyme. After enzymatic hydrolysis, a first centrifugation eliminates the non-hydrolyzed residues. A second centrifugation allows the removal

of fat, which may make protein hydrolyzates more vulnerable to environmental conditions such as oxidation and inhibit the growth of certain bacterial strains. In order to get protein hydrolyzates with different DH, the method described by Kristinsson and Rasco (2000) was used. Enzyme/Substrate ratios (E/S) were in ascending order: 0.087, 0.69 and 0.87, the same amount of substrate was used except that the amount of enzyme was increased. The kinetics of hydrolysis of Sardinelle proteins are shown in Fig. 2.

All curves have the same look even when E/S ratio was varied. However, increasing the E/S ratio increases the DH value. The kinetics of enzymatic hydrolysis are characterized by a rapid initial phase during which a large number of peptide bonds is hydrolyzed. After this phase, the DH is stabilizing to reach maximum values. The evolution of the DH during the time seems to be identical to that obtained with casein (Mahmoud et al., 1992). Similar results were described by Shahidi et al., (1995) showing high concentration of fish soluble peptides produced during the initial phase of hydrolysis and remained in the reaction mixture. This accumulation of soluble peptides reduced the rate of hydrolysis. However, the content of soluble peptides can continue to increase while increasing the enzyme concentration and extending the reaction time (Surowka and Fik, 1994). Three different protein hydrolyzates which are named SPH1, SPH2 and SPH3 with respective DH of 12, 20 and 26% have been developed.

3.2. Chemical characterization of sardinelle protein hydrolyzates

The chemical composition of the different protein hydrolyzates (SPH1, SPH2 and SPH3) is shown in Table 2.

It appears that all protein hydrolyzates contain a suitable protein content ranged between 60 and 63%. However, this protein content remains lower than tryptone and requires further optimization to achieve the proteins levels as reported for commercial peptones (81.25 %) (Dufossé et al., 2001). Sardinelle protein hydrolyzates have a low moisture content not exceeding 5%, allowing its storage for longer durations. Fat content is lower than the initial raw material, which shows the effectiveness of centrifugation in eliminating fat. Interestingly, the fat content in protein hydrolyzates is inversely related to DH. This can be explained by the release, following the reaction of hydrolysis, of fat globules trapped in proteins from fish muscle (Kristinsson and Rasco, 2000). We also find that protein hydrolyzates are especially rich in ash depending on hydrolysis duration (value varied from 19.22% for SPH1 to 22.30% for SPH2). This can be explained by the fact that during the hydrolysis reaction, the pH is kept constant by continuous addition of NaOH allowing the enhancement of ash content in the final product in the form of sodium (Kristinsson and Rasco, 2000).

3.3. Microbial growth test on Sardinelle protein hydrolyzates

Prepared hydrolyzates were tested for the production of biomass by *E. coli* and *S. cerevisiae*. The results are listed in Fig. 3. The biomass production (Fig. 3a) shows that *E. coli* grows well on different Sardinelle protein hydrolyzates exceeding that obtained on control medium (LB medium). The best production is obtained with protein hydrolyzates SPH1 and SPH2, but the difference between the two protein hydrolyzates was not statistically significant at $P<0.05$.

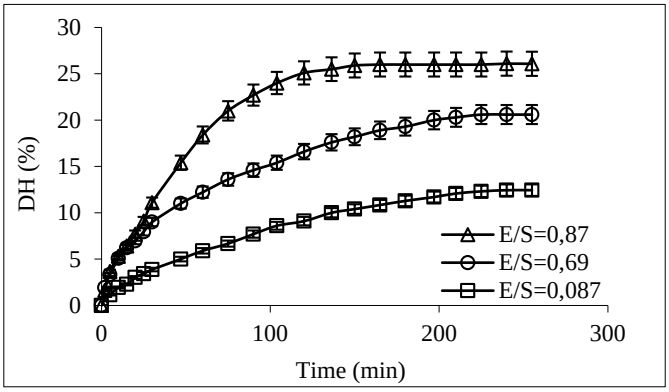
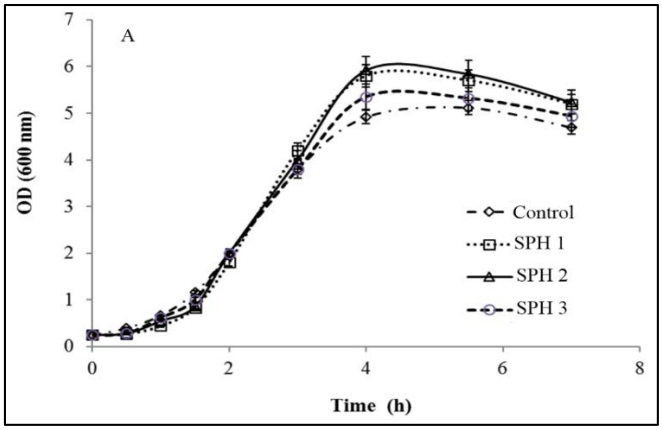


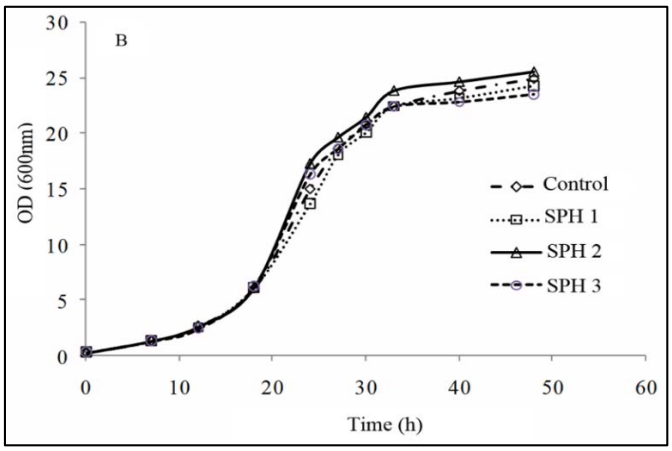
Fig. 2. Effect of Enzyme/Substrate ratio on the degree of hydrolysis of Sardinelle protein using enzyme preparation of *B licheniformis* NH1

Table 2. Chemical composition of different Sardinelle protein hydrolyzates (g/100 g product)

	Dry matter	***Ash***	***Protein***	***Lipids***
Whole sardinelle	29.06±0.31	6.12±0.53	14.12±1.89	9.35±0.17
Cooked sardinelle	13.49±0.22	2.09±0.46	9.05±1.21	2.12±0.72
SPH1 (12 %)	95.17±2.13	19.22±2.11	60.78±4.11	8.96±2.46
SPH2 (20 %)	96.21±2.43	21.49±2.48	61.63±3.92	7.71±2.21
SPH3 (26 %)	97.73±2.76	22.30±2.83	63.13±4.78	6.77±1.86
Tryptone (Organotechnie SA)*	92.62±1.23	8.5±0.98	81.25±0.32	2.5±1.56



(a)



(b)

Fig. 3. Microbial growth kinetics in different SPH media: (a) *E. coli*; (b) *S. cerevisiae*

However, there is a significant difference between hydrolyzates (SPH1 and SPH2) and the control medium. The lower growth obtained in SPH3 may be related to its highest DH allowing the presence of more minerals that may inhibit *E. coli* growth.

According to the profile of growth curves of *S. cerevisiae* (Fig. 3b), all the protein hydrolyzates tested allows good growth of the studied strain. However, statistical analysis shows no significant difference between control medium and protein hydrolyzates-based media. Our results confirmed the fact that fish by-products provide an excellent source for microbial growth, which can be exploited in producing various metabolites such as protein hydroysate and enzymes.

3.4. Proteases production on sardinelle protein hydrolyzates

According to Fig. 4, media containing fish protein hydrolyzates enhanced protease synthesis by *B. cereus* SV1 while compared to M2 medium. This indicates that the strain can obtain its nitrogen requirement directly from protein hydrolyzates. Highest protease production was achieved with SPH2 and was the same as that obtained with control medium. For *V. metschnikovii* J1 strain, the protease synthesis in media containing SPH as nitrogen source were lower than that obtained with the control medium (Fig. 5). The higher level of proteolytic activity was observed on medium containing SPH1 (DH = 12%): it is about 6454.545 U/mL. By increasing the DH up to 20%, the proteolytic activity decreased to 2409 U/mL. The lower activities with protein hydrolyzates with high HD could be attributed to the apparition of peptides which could affect bacterial growth and/or protease synthesis.

Similarly, protease production by *B. amyloliquefaciens* An6 (Fig. 6) increased with the increase of DH of SPH, and enzyme activity was slightly higher with SPH3 (2005.5 U/mL) than that obtained with M4 medium containing yeast extract as nitrogen source (1825.5 U/mL). This suggests that the strain can obtain its nitrogen requirement more efficiently from amino acid and low molecular weight peptides than high molecular weight peptides.

Experiment conducted with *P. aeruginosa* MN7 (Fig. 7) with SPH at a concentration of 10 g/l, showed the production of similar amount of protease with SPH1 and SPH2 protein hydrolyzates (4232 and 4235 U/mL, respectively) as that obtained with M5 medium containing casein peptone (4210 U/mL). Nevertheless, with SPH3 protein hydrolyzate, the level of protease synthesis was slightly higher than obtained with M5 medium. Therefore, it appears that

sardinelle protein hydrolyzates are suitable for the production of protease by *P. aeruginosa* MN7, which can obtain its carbon and nitrogen sources from casein peptone as well as SPH. The obtained results on microbial protease production confirmed what was reported by various studies. For example, an acceptable level of protease production was obtained for *Pseudomonas aeruginosa* MN7 and *Bacillus subtilis* growing in fish heads and viscera powder based media (Ellouz et al., 2001;Triki-Ellouz et al., 2003).

In the same context, it was demonstrated that peptones obtained from fish viscera of rainbow trout, swordfish , squid and yellow fin tuna species can be used as growth media for vibrio species (*Vibrio anguillarum* and *Vibrio splendidus*) allowing higher level of protease production compared to commercial peptone-based media (Vázquez et al., 2006).

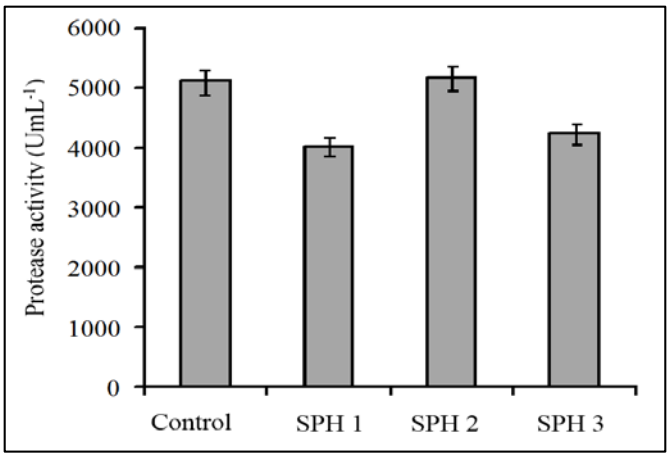


Fig. 4. Protease production by *B cereus* SV1 in different SPH media

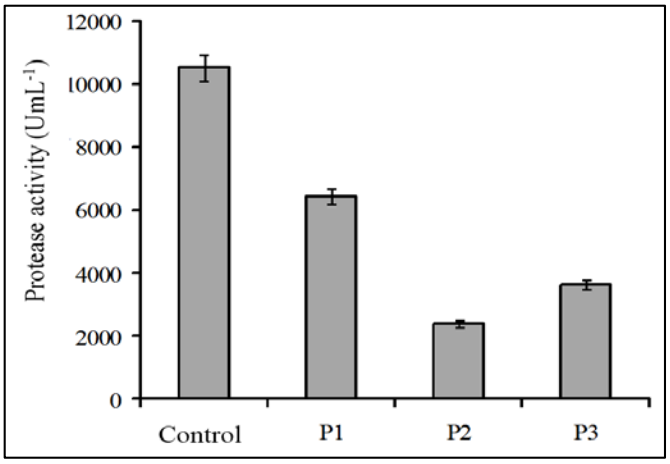


Fig. 5. Proteases production by *V metschnikovii* J1 in different SPH media

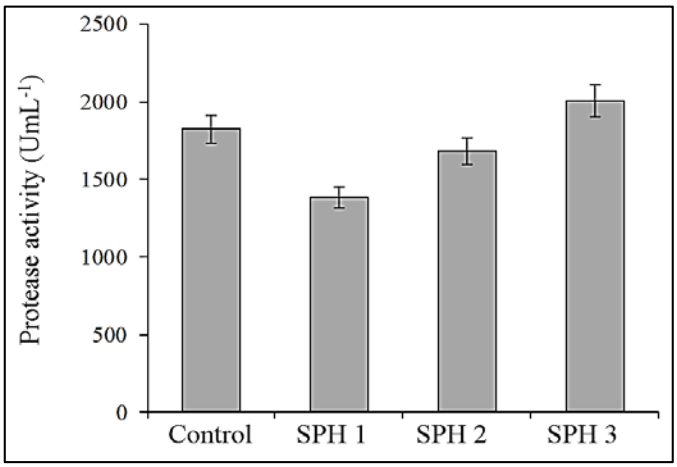


Fig. 6. Proteases production by *B amyloliquefaciens* An6 in different SPH media

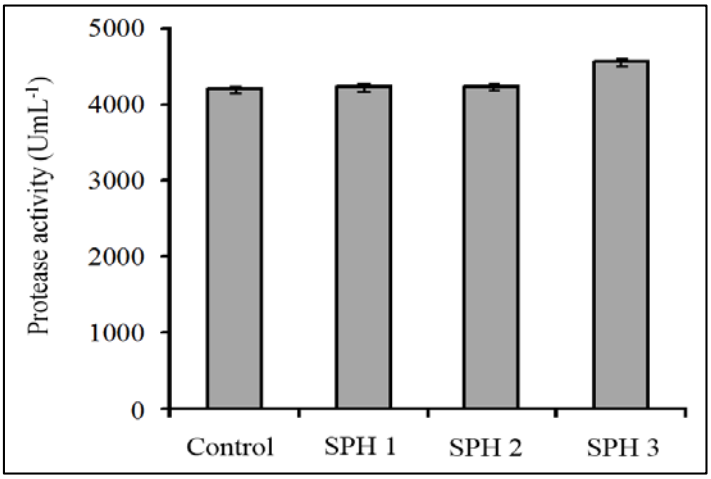


Fig. 7. Proteases production by *P aeruginosa* MN7 in different SPH media

In our preparation method of of Sardinelle protein hydrolyzates, we applied a second centrifugation in order to remove fat. As reported by Esakkiraj et al. (2009), defatted products enhances the protease production as reported for *Bacillus cereus* grown on defatted tuna waste. The improvement of protease production in the defatted fish-based media could be related to the lipid-free nature of product, which could support the microbial protease synthesis than other types of nitrogen source. The same deduction was reported for lipase production by by *Rhizopus oryzae* (Ghorbel et al., 2005). However, is very important to note that optimal protease production is controlled by various growth parameters including the medium composition (carbon and nitrogen sources, mineral salts), agitation, temperature, pH etc.

4. Conclusions

Protein hydrolyzates with different degrees of hydrolysis (12, 20 and 26%) were prepared from sardinelle heads and viscera. The protein content of these protein hydrolyzates is between 62.7 and 66%. Interestingly, microbial growth experiments showed that these hydrolyzates are an excellent nitrogen source for the growth of various microbial strains (*E. coli*, *S. cerevisiae*, *B. cereus* SV1, *B. licheniformis* NH1, *B. amyloliquefaciens* An6 and *P. aeruginosa* MN7).

Interestingly, it was demonstrated that these protein hydrolyzates offer good potential for microbial protease production. This proved the validity of these protein hydrolyzates as nitrogen and/or carbon sources for biomass and protease production. Furthermore, the use of protein hydrolyzates obtained from processing by-products may reduce considerably the cost of enzyme synthesis.

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