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Use of hydrolysates from yellowfin tuna *Thunnus albacares* fisheries by-product as a nitrogen source for bacteria growth media

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Abstract

Hydrolysates of tuna head, using commercial enzymes Alcalase® (Alc) and Protamex™ (Prot), were tested as growth media for the bacterial strains of *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Streptococcus faecium*, *Listeria monocytogenes*, *Bacillus subtilis*, *Bacillus licheniformis* and *Micrococcus lysodeikticus*, as an alternative to commonly used complex sources (peptones and/or extracts). Peptones from the enzymatic hydrolysis by Alcalase and Protamex were used instead of standard peptones used in commercial media. All bacteria were cultured on traditional standard media as controls, and their growth was compared to ones grown on experimental media containing Alc- and Prot-peptone instead of the standard peptone. Peptones produced by Alcalase and Protamex had obtained 34 and 19% degree of hydrolysis, respectively. The results showed that both the Alcalase and Protamex peptones were more effective at promoting bacterial growth than the standards used in traditional media, while the peptone from Alcalase, with a higher percent of hydrolysis, was more effective than the peptone from Protamex, also in terms of biomass production, Thus the choice of the proteolytic enzymes used to produce the fish hydrolysates had a considerable impact on the performance of the resulting hydrolysate.

Keywords: Bacteria, Fish hydrolysate, Growth media, Peptones, Alcalase, Protamex

Introduction

The biotechnological fermentation industries show an increasing demand for microbial growth media for which usually the most expensive component is the nitrogen source. Many microbes may utilize a cheap inorganic nitrogen source, such as ammonium and even nitrite salts (Aspmo et al. 2005a). Even microbes synthesizing all their primary metabolites *ex novo* can be stimulated to grow faster and to higher cell densities in a rich complex medium due to the presence of more easily convertible nitrogen and favorable growth factors (Burkovski and Kramer 2002). When the defined media are formulated to include all the necessary compounds for optimal growth, it could become quite expensive, limiting their use in a commercial process (Aspmo et al. 2005a).

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Peptones are derived primarily from products of bovine or porcine origin, such as meat, internal organs, gelatin and milk, but also from plants and yeasts, which are the most expensive media ingredients (Aspmo et al. 2005a). Because of recent outbreaks of bovine spongiform encephalopathy (BSA) and a growing demand for raw materials that are 'kosher' approved and certified free of swine flu, peptones of a non-meat origin are becoming increasingly important (Aspmo et al. 2005a; Safari et al. 2009). Every year over 132 million tons of fish are harvested, of which 29.5% is converted into fish meal (FAO 2006). Possibly more than 50% of the remaining fish tissue is processed as waste and not used as food. The use of fish materials as source of nutrients for microbes has been reported as early as 1949 (Tarr and Deas 1949). Since then, several attempts to explore the use of fish peptones as component of microbial growth substrates have been reported (Gildberg et al. 1989; Dufosse et al. 2001; Vazquez et al. 2004; Aspmo et al. 2005a, b; Horn et al. 2007; Vazquez and Murado 2008; Vazquez et al. 2008; Safari et al. 2009). Fish peptones are usually produced by hydrolysis with exogenous enzymes (Dufosee et al. 2001; Aspmo et al. 2005c; Ovissipour et al. 2009; Safari et al. 2009).

Yellowfin tuna *Thunnus albacares* is one of the most important pelagic species in Iran with an annual catch of 41 000 metric tons (IFO 2006). In this study, the potential of two different peptones from yellowfin tuna head using Alcalase 2.4 L and Protamex™ on growth of some bacterial strains was studied in comparison with those used in commercial standard media.

Materials and methods

Yellowfin tuna were obtained from the fish processing company in Sari, Iran, which were caught in Bandar Abbas, south of Iran, and immediately frozen at -20ºC once on board. The heads from the frozen fish were taken and immediately transferred to the university laboratory; here, they were minced twice using an industrial mixer with medium speed (Jaltajhiz, Tehran, Iran, 5 mm in mesh size) and mixed with distilled water (1:1 w/v). The preparation of peptones was performed according to our previous studies (Ovissipour et al. 2009; Safari et al. 2009). Briefly, the mixture was heated at 85°C in a water bath (W614-B, Fater Rizpardaz, Tehran, Iran) for 20 min to deactivate endogenous enzymes. In separate vessels, Alcalase 2.4 L ("Alc") and Protamex™ ("Prot") (Novozymes, Bagsvaerd, Denmark) were added to the substrate at 1.5% (v/w) and 1.5% (w/w), respectively. All reactions were carried out in 250 ml glass Erlenmeyer flasks, in a shaking incubator (GTSL20, Jaltajhiz, Tehran, Iran) with constant agitation (150 rpm orbital shaking) at 55°C for 24 h at neutral pH.

After 24 h, the reactions were terminated by denaturation of the enzymes by heating at 95°C in a water bath for 20 min. The reaction mixtures were centrifuged (8000 rpm/ 20 min) using 50 ml tubes at 4°C in a Hettich D-7200 (Tuttlingen, Germany) centrifuge. The supernatants, containing the soluble peptones, were stored at -20ºC until the time of use for the formulation of culture media.

The bacteria used in this study were selected from PTCC (Persian Type Culture Collection) and ATCC (American Type Culture Collection). The bacterial culture conditions are presented in Table 1. The lyophilized bacteria were cultured on appropriate media at optimum temperature.

Table 1. Microorganisms employed

Strain	References	Standard medium	pH	Temp (°C)
Pseudomonas putida	PTCC* 1694	King Agar	7.1	32
Pseudomonas aeruginosa	PTCC 1555	King Agar	7.1	32
Streptococcus faecium	PTCC 1237	Blood Agar	6.8	35
Listeria monocytogenes	PTCC 1163	Blood Agar	6.8	30
Bacillus subtilis	PTCC 1156	Plate Count Agar	7	35
Bacillus licheniformis	PTCC 1331	Plate Count Agar	7	35
Micrococcus lysodeikticus	ATCC 4698	Tryptone Soy Agar	7.3	35

Notes:
*PTCC: Persian Type Culture Collection, ATCC: American Type Culture Collection

The composition of the media is summarized in Table 2. It should be noted that in all media the protease materials in the standard medium were replaced by the same amount of peptones from the Alc and Prot hydrolyzed wastes in the experimental media, and correspondingly the same was done with all other ingredients. In all cases, the initial pH of the medium was adjusted to the recommended pH (Table 1) using 0.2 N HCl, and the solutions were sterilized at 121°C for 15 min at the pressure of 1.1 atm using autoclave (KI-40D, ALP, Japan). The tuna head protein hydrolysates using Alc and Prot contained 80 and 75 mg/ml protein

according to the Biuret method (Layne 1957), using bovine serum albumin as the standard protein. All other ingredients in the media were obtained from Merck (Darmstadt, Germany).

Table 2. Standard media composition[a]

	TSA	BA	KA	TC
Sodium chloride	5.0	5.0	_	_
Glucose	_	_	_	1.0
Agar agar	15.0	15.0	10.0	14.0
Magnesium sulfate	_	_	1.5	_
Tripotassium phosphate 3-hydrate	_	_	1.8	_
Protease peptone	_	_	20.0	_
Heart extract & peptone	_	20.0	_	
Casein peptone	_	_	_	5.0
Soy meal peptone	15.0	_	_	2.5
Yeast extract	5.0	_	_	_

Notes:
[a] All ingredients correspond to (g/l).

The soluble proteins in the fish hydrolysates were measured in the supernatant following centrifugation by the Biuret method (Layne 1957), using bovine serum albumin as the standard protein. The absorbance was measured at 540nm with a UV/vis spectrophotometer (Cecil 1020, England). The total nitrogen in raw materials and peptones was measured using Kjeldahl (AOAC 2005). The ash content was calculated after ashing at 550°C for 16 h (AOAC 2005). All analyses were performed using triplicate samples ($n=3$).

The data from the chemical analyses were subjected to one-way analysis of variance (ANOVA) and the data of the degree of hydrolysis were analyzed by unpaired *t*-test using SPSS statistical software, release 16.0 (SPSS Inc., Chicago, IL, USA). Duncan's new multiple range test was carried out in order to determine the significant differences between the means at the 5% probability level.

Results and discussion

The growth of seven different bacteria, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Streptococcus faecium*, *Listeria monocytogenes*, *Bacillus subtilis*, *Bacillus licheniformis* and Micrococcus *lysodeikticus*, cultured on traditional media, was compared with that of the bacteria cultured on media containing Alc- and Prot-peptones.

The chemical composition of the nitrogen sources is presented below in Table 3. The two fish protein hydrolysates using Alc and Prot had a nitrogen content of 12.8% and 11.7%, respectively. The results showed that the peptones produced by Alc had more nitrogen than the ones produced by Prot and the degree of hydrolysis of the resulting tuna head peptones produced by Alc (34 ± 5%, $n=3$) was higher than the ones produced by Prot (19 ± 3%, $n=3$), which differences observed were significant ($P < 0.05$). There was not any significant difference ($P > 0.05$) between nitrogen sources with respect to the total nitrogen content. This study showed that, despite using the same raw material, the choice of proteolytic enzyme clearly affects the bacterial growth performance.

Table 3. Chemical specification of nitrogen sources*

Name	Total nitrogen (%)	Ash (%)	Source
Casein peptone	13.15 ± 1.52[a]	6.6	Casein
Yeast extract	11.63 ± 0.46[a]	12.8	Yeast
Soytone	9.26 ± 2.8[a]	12.43	Soy
Proteose peptone	14.32 ± 3.5[a]	ND	-
Heart extract and Peptone	ND[**]	ND	-
Alc	12.84 ± 2.33[a]	5.6	Tuna head
Prot	11.68 ± 1.03[a]	5.8	Tuna Head

Notes:
[*] Values represent % of dry matter. Values represented means ± SE ($n=3$), Values in column with different superscripts are significantly different at $\alpha= 0.05$
[**] ND= Not determined.

The superior performance of peptones obtained with Alc may be explained in several ways. Alc leads to optimal uptake of the available amino acid resources, because this endoprotease activity is the most complementary to the bacterial proteolytic and peptide-uptake systems, and the hydrolysates produced by Alc show a decrease in high molecular weight fractions and increased solubility (Kristinsson and Rasco 2000). Aspmo et al. (2005b) and Ovissipour et al. (2009) demonstrated that peptones produced by Alc have a higher content of short peptides. Another explanation is related to the higher degree of hydrolysis in Alc-peptones (34%) in comparison with Prot-peptones (19%). It seems that the higher degree of hydrolysis in peptones from Alc would accelerate the absorption of peptones from the media (Gildberg et al. 1989).

Table 4. The growth of bacteria in different media after 24 h[a]

Bacteria	Medium[b]		
	King Agar	Alc	Prot
Pseudomonas putida	$(23 \pm 4)\times10^5$	$(52 \pm 7)\times10^5$	$(49 \pm 3)\times10^5$
P. aeruginosa	$(13 \pm 8)\times10^5$	$(35 \pm 13)\times10^5$	$(18 \pm 8)\times10^5$
	Blood Agar	Alc	Prot
Streptococcus faecium	$(28 \pm 11)\times10^4$	$(11 \pm 9)\times10^5$	$(82 \pm 12)\times10^4$
Listeria monocytogenes	$(63 \pm 4)\times10^4$	$(83 \pm 6)\times10^4$	$(74 \pm 8)\times10^4$
	Plate count Agar	Alc	Prot
Bacillus subtilis	$(32 \pm 2)\times10^6$	$(74 \pm 11)\times10^6$	$(43 \pm 8)\times10^6$
B. licheniformis	$(45 \pm 6)\times10^5$	$(83 \pm 14)\times10^5$	$(59 \pm 7)\times10^5$
	TSA	Alc	Prot
Micrococcus lysodeikticus	$(34 \pm 8)\times10^5$	$(53 \pm 13)\times10^5$	$(43 \pm 6)\times10^5$

Notes:

[a] Values represent Colony Forming Unit (CFU)/ml

[b] Values represented means ± SE ($n=3$), Values in rows with different potentials are significantly different at $\alpha= 0.05$

The capacity of bacteria for colony forming on different nitrogen sources was evaluated by comparing their growth on standard and FPH media. The results of bacterial growth after 24 h showed that the peptones from Alc and Prot hydrolyzed wastes were more effective than those of the traditional media. The colony morphology and size were similar in all kinds of media (not shown). The results of this study revealed that FPH can enhance the growth of all seven bacteria (Table 4). Similar results were obtained by many researchers (Vazquez et al. 2004; Aspmo et al. 2005a, b; Vazquez and Murado 2008; Vazquez et al. 2008; Safari et al. 2009). They found that peptones derived from marine source used for nitrogen in bacterial media have positive effects on bacterial growth. Safari et al. (2009) found that lactic acid bacteria (LAB) grew better in experimental media which contained Alc and Prot peptones than in traditional media.

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