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ORIGINAL ARTICLE

Autohydrolysed *Tilapia nilotica* Fish Viscera as a Peptone Source in Bacteriocin Production

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Abstract Fish processing generates large amounts of solid and liquid wastes. Many different by-products have been produced from fish processing wastes. Studies on solubilization of Bolti fish (*Tilapia nilotica*) viscera by endogenous enzymes at different pHs are described. Hydrolysis reactions were conducted with freshly thawed viscera utilizing an initial temperature gradient and terminated at various time points by heat inactivation of the enzymes. Various peptones obtained from hydrolysed visceral homogenates of Bolti fish residues showed their suitability for promoting the growth of lactic acid bacteria (mainly *Lactobacillus sake* Lb 706), microorganisms with particularly complex nutritional requirements especially peptidic sources. The assay of several treatments with *L. sakei* Lb 706, producer of the bacteriocin sakacin A, demonstrated that optimum conditions for biomass and bacteriocin production only imply a brief autohydrolysis at room temperature. The results showed that the Bolti fish hydrolysates gave remarkable results to those found in costly commercial media, specifically recommended for culturing and large-scale production of lactic acid bacteria.

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Introduction

Fish sources once appeared to be inexhaustible and by-products arising out of fish processing were looked as worthless garbage and discarded without an attempt of recovery [1]. Fish processing generates large amounts of solid and liquid wastes. Many various by-products have been produced from fish processing wastes. However, fishery by-products are typically feeds and fertilizers that have a low economic value. There is growing interest in obtaining valuable biochemicals and pharmaceuticals from fishery wastes [2]. One way to add value to proteinaceous fish waste is to convert it to hydrolysates. Such hydrolysates have a range of potential applications, e.g. as ingredients in animal feed [3], human food [4], as fertilizer [5] or as peptone source in microbial growth media [6].The term ‘‘peptone’’ is used for protein hydrolysates that are soluble in water and not heat coagulable [7]. The market price of peptones is higher than that of the usual fishery by-products, and development of peptones may thus be of significant value. Further, fish processing wastes including viscera have been reported to be good substrates for lactic acid fermentation [8]. Lactic acid bacteria (LAB) are an important microbial group, either as natural microbiota, or inocula added under controlled conditions. Among the bioactive molecules produced by LAB are bacteriocins. Bacteriocins are peptides with antimicrobial activity and have interest in alimentary industry as they are innocuous, sensitive to digestive proteases, and do not change the organoleptic properties of the food [9]. One problem associated with the production of LAB and bacteriocins on

an industrial scale is their demand for diversified peptidic sources, which costly commercial media resolve by including products such as bactopeptone, triptone, meat extract or yeast extract in their formulations. Therefore, the use of low-cost protein fractions will bring about an effective reduction in large-scale production costs. Furthermore, if food waste such as fish viscera is used to obtain those protein fractions, a productive cycle is closed: recycling of a pollutant waste and obtaining valuable products (e.g. LAB and bacteriocins) useful for preservation of foodstuff.

Based on these considerations, this study examines the suitability of autohydrolysates of Bolti fish viscera as a peptone source in formulating microbiological media (FP). The microorganism tested was *L. sakei* Lb 706, one of the most fastidious (lactobacilli) and producer of one of the important bacteriocin used in meat applications, sakacin A. On the other hand, three microbiological media were used as terms of comparison as follows: (1) formulated medium with commercial bactopeptone as fish peptone replacement (BP), (2) commercial Difco MRS medium (MRS-D) and commercial Oxiod MRS medium (MRS-O). As criteria for comparison, the biomass and bacteriocin production for deferent incubation times were used. If these biological compounds can be recovered, it would serve the dual purpose of recovery of these biomolecules and reducing the pollution problems associated therewith.

Materials and Methods

Preparation of Fish Autohydrolysates

Raw Materials: Bolti fish, *Tilapia nilotica*, were purchased from the Egyptian local market and the viscera were quickly removed by hand, and then stored in sealed plastic bags for a maximum period of 15 days at −20°C. The viscera masses were grinded with 10% (v/w) distilled water, then the homogenate divided into four various portions (all of them have pHs of 6.16 to 6.39). The pH of each portion was adjusted using 4 N HCl to specific values of pH 6.2, 5.2, 4.2, and 3.2 afterwards, the homogenates were incubated for 8, 18, 32 and 47 h at 20°C, with orbital shaking at 100 rpm. Samples were taken after each incubation period and the autohydrolysates were heated at 85°C for 20 min to inactivate the endogenous enzymes [10] and to facilitate the removal of the fat present in fish material [11]. Then the heated samples were centrifuged at 6,000 rpm for 15 min to obtain the corresponding sediments and supernatants. The supernatants (fish peptones) were typified determining the levels of total protein and then stored at −20°C until used in the formulation of culture media.

Microbiological Methods

L. sakei Lb 706 was used as the producer of sakacin A bacteriocin [12]. *L. sakei* NCDO 2714 was used as sakacin A-sensitive indicator organism to determine bacteriocin activity. *L. sakei* Lb 706 and *L. sakei* NCDO 2714 were kindly provided by Prof. Ulrich Schillinger (the Institute of Hygiene and Toxicology, BFE Karlsruhe, Germany) and Prof. Ingolf Nes (Laboratory of Microbial Gene Technology, Agriculture University of Norway), respectively. Strains were stored at −80°C in MRS medium, both containing 25% (vol/vol) glycerol as a cryoprotectant. To produce fresh cultures, the strains were propagated twice at 25 and 30°C for 14–16 h before experimental use, respectively. Solid medium was prepared by adding 1.5% (wt/vol) agar (Difco Laboratories) to the broth. The overlays used for estimation of bacteriocin titers were prepared with 0.75% (wt/vol) agar. The composition of the media is shown in Table 1. For comparative purposes, a medium (BP) was used where the fish peptones were replaced by a commercial bactopeptone solution with an equivalent protein level, as well as MRS commercial media. In all cases, initial pH was adjusted to 7.0 and solutions were sterilized at 121°C for 15 min. Micro-organisms were grown in 300 ml Erlenmeyer flasks with 200 ml of medium at 25°C, with 100 rpm orbital shaking. The cultures were carried out in triplicate. At pre-established times, each culture was divided into two aliquots. The first aliquot was

Table 1 Composition of culture media used in microbiological assays (g l^{-1})

Component	FP	Media with non-fish peptone		
		BP	MRS-D	MRS-O
Glucose	20	20	20	20
Yeast extract	2	2	5	4
Sodium acetate	3	3	5	5
Ammonium citrate	2	2	2	2
K_2HPO_4	2	2	2	2
$MgSO_4$	0.20	0.20	0.10	0.20
$MnSO_4$	0.05	0.05	0.05	0.05
Tween 80	1.0	1.0	1.0	1.0
Meat extract	–	–	10	–
Bactopeptone	–	10	–	10
Proteose peptone no. 3	–	–	10	–
Beef extract	–	–	10	–
Lab-lemco powder	–	–	–	8
Fish peptone protein (lowery)	10	–	–	–

FP medium prepared with fish peptones

BP medium prepared with commercial bactopeptone

MRS-D medium prepared with commercial formulation from DIFCO

MRS-O medium prepared with commercial formulation from OXOID

centrifuged at 6,000 rpm for 15 min, and the sediment washed twice and re-suspended in distilled water to the adequate dilution to measure the absorbance at 700 nm. The dry weight can then be estimated from a calibration curve. The corresponding supernatant was used for the determination of protein contents [13]. The second aliquot was used for the extraction and quantification of bacteriocin. All assays were carried out in duplicate.

Bacteriocin Activity Assay

The culture supernatants were assayed for bacteriocin activity by the spot on lawn technique with MRS agar using *L. sakei* NCDO 2714 as indicator strain [14]. Indicator lawns were prepared by adding 0.125 ml of ten times diluted overnight culture to 5 ml of MRS soft agar (0.75%). The contents of the tubes were gently mixed and poured over the surfaces of pre-poured MRS agar plates. Bacteriocin samples were sterilized by passage through a 0.45 µm cellulose acetate filter. Serial twofold dilutions were carried out in the same medium as used for the growth of the indicator strain. Activity was quantified by taking the reciprocal of the highest dilution that exhibited a clear zone of inhibition and was expressed as activity units (AU) per milliliter of culture media. The titre of the bacteriocin solution, in AU/ml, was calculated as (1000/d) D, where D is the dilution factor and d is the dose (the amount of bacteriocin solution pipetted on each spot) [14]). To avoid errors in bacteriocin activity values, the same person made all observations.

Results

The proposed scheme described here in hydrolyzing viscera leads to the recovery of 0.7–0.8 l of peptonated solution per kg (fresh weight). The progress of the hydrolysis slightly increases the volume recovered, in equivalent proportions, up to 10–15% after 47 h. Samples of LAB cultures were taken at times corresponding to the logarithmic phase and the beginning of the asymptotic phase (8 and 16 h). Although both groups of samples led to mutually results, only values obtained at the beginning of the asymptotic phase are compared, these being more representative of the productions studied (16 h). The regulatory deduced from the results are: In the most productive cultures with fish peptones: (a) the biomasses surpassed those obtained with BP and MRS media, (b) the sakacin A production by *L. sakei* Lb 706, always surpassed that obtained with BP and surpassed or equaled that obtained with commercial MRS, (c) the highest productions of biomasses with fish peptones processed at different pHs were obtained at the lowest hydrolysis time (0, 8 and 18 h)

L. sakei Lb 706 was grown on 23 different media containing 20 out of 23 tested peptone hydrolysates from Bolti viscera at various pHs. The amount of peptone added were based on protein content, since this allows the most straight forward comparison of relationships between protein content and performance. Table 1 shows clear differences between the various fractions and subfractions. Samples of the same subfraction, were taken at various time points during hydrolysis process. The samples taken at 8, 18 and 32 h showed slightly higher variations in protein content compared to those obtained at 0 and 47 h. Approximately, 47 h-hydrolysates had very similar total protein content in all samples except for sample processed at pH 6.2. We previously observed that hydrolysis is more or less completed after 47 h under the conditions used at various pHs (Table 2).

The growth of *L. sakei* Lb 706 as biomass (Table 3) show that the highest cell density was achieved using peptone hydrolysate at pH 3.2 followed by pH 6.2, 5.2, respectively, at different process times. Generally, the fish based peptones performed very well, and all pHs did better than the other peptones when it comes to biomasses production. Of the non-fish peptones media from Difco was one of the best and BP media was the worst in terms of biomass production.

Table 2 Total protein content (g l^{-1}) of fractions of fish viscera hydrolysates at various pHs

Hydrolysis time (h)	pH 6.2	pH 5.2	pH 4.2	pH 3.2
0	24.4	23.79	23.97	24.4
8	28.2	26.11	23.41	18.76
18	28.1	24.06	24.66	26.06
32	26.9	24.0	26.21	23.5
47	27.46	30.2	30.5	30.05

Table 3 Production of *L. sakei* Lb 706 biomass on viscera hydrolysates at the beginning of the asymptotic phase (16 h)

Hydrolysis time (h)	Biomass (g l^{-1}) at various initial pHs of hydrolysis			
	pH 6.2	pH 5.2	pH 4.2	pH 3.2
0	2.24	2.22	2.20	2.23
8	2.12	2.0	1.34	2.49
18	2.01	1.79	1.41	2.05
32	1.7	1.39	1.65	2.33
47	1.6	1.32	1.83	2.66
MRS-D	2.0	–	–	–
MRS-O	1.45	–	–	–
BP	0.95	–	–	–

BP medium prepared with commercial bactopeptone; *MRS-D* medium prepared with commercial formulation from Difco; *MRS-O* medium prepared with commercial formulation from Oxoid

Table 4 Production of sakacin A on viscera hydrolysates at the beginning of the asymptotic phase (16 h)

Hydrolysis time (h)	Bacteriocin activity (AU/ml) at various initial pH of hydrolysis			
	pH 6.2	pH 5.2	pH 4.2	pH 3.2
0	6400	6400	6400	6400
8	3200	1600	3200	2400
18	1600	800	4800	1600
32	3200	12800	4800	1600
47	12800	19200	6400	1600
MRS-D	6400	–	–	–
MRS-O	4200	–	–	–
BP	3200	–	–	–

BP medium prepared with commercial bactopeptone; *MRS-D* medium prepared with commercial formulation from Difco; *MRS-O* medium prepared with commercial formulation from Oxoid

For bacteriocin production of sakacin A from *L. sakei* Lb 706, differences in levels and production rates were particularly notable (Table 4) ranged from 3,200 to 6,400 AU/ml using commercial MRS and BP. Adding a 32 h-hydrolysate fraction at pH 5.2 increased the activity to 12,800 AU/ml while extend the hydrolysis to 47 h at eh same pH increased the bacteriocin activity to over 19,200 AU/ml. Significant differences were noticed even between commercial MRS media. In general terms, the results described above lead to the following conclusions: (1) hydrolysates extract fractions were the best in biomass production compared to other treatments, (2) the performance of the various fish peptone fractions from Bolti fish varied considerably and (3) the order of performance varied with hydrolysis pH and time. Generally, fish peptone from Bolti fish showed performances in the same range as the commercial peptones.

Discussion

LAB are fastidious bacteria and thus good indicators of the lot to lot variance in growth media. Also, a medium supporting growth of LAB is probably good medium for many other microorganisms with similar or less nutritional requirements. In this study a type strain belonging to what is regarded as the most fastidious genus of the gram positive bacteria. *L. sakei* is known to have the most fastidious nutritional requirement of all the lactobacilli [15]. Hence a medium supporting growth of *L. sakei* should permit growth of all other lactobacilli. One possible explanation for the good performance of the fish peptones could be their amino acid composition with high levels of branched amino acids such as, leucine, valine and isoleucine which are essential for *L. sakei* strain [16]. Obviously, the amino acid composition alone does not explain the better performance of the fish peptones, since these peptones themselves show quite different performances under different pHs with process time which might support different endo proteases which might lead to different degree of hydrolysis regarding detailed composition of hydrolysate such as, peptide length, peptide sequences and amount of free amino acids. Therefore, its conceivable that Bolti fish peptone hydrolysates with its different content of peptides and amino acids suit the lactobacillal transport and processing machineries [17].

There are several other factors that may contribute to observed differences in performance of the Bolti fish peptones at different pHs and time. The presence of vitamins is essential for the *L. sakei* type strain [2] such as riboflavin (vitamin B2), nicotinic acid (niacin or vitamin B3), and D. panthothenic acid (vitamin B5). These water soluble compositions are present in varying quantities in fish viscarea [18]. The fact that growth was achieved with all fractions of Bolti fish peptones, prove that these vitamins are present in sufficient quantities in different fractions of fish hydrolysates.

Furthermore, the highest productions of biomasses with Bolti fish peptones processed at different pHs were obtained at the shortest hydrolysis time. The suitability of the short hydrolysis times is somewhat surprising, as initially a greater peptidic diversity and nutritional capacity for microorganisms could have been expected with more advanced hydrolysates. The results (repeatedly confirmed) may be interpreted by admitting the exact opposite, or supposing that hydrolysis affects peptides of interest, which are free at the initial stage of the system or, finally, that during the process of hydrolysis biogenic amines are released [19] which could inhibit the growth of certain microorganisms. The promising results obtained with *L. sakei* are of interest because lactobacilli (and other LAB) have a GRAS (generally regarded as safe) status, because some LAB have potential as probiotics, and because some LAB may be exploited as (in situ) food-grade cell factories and delivery vehicles for e.g. antigens, antibodies and growth factors [20]. Thus the ability to effectively grow bacteria on media free of compounds derived from meat sources or from plant sources (e.g. soya) potentially containing genetically modified materials is of considerable interest [16].

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