Fish protein hydrolysate production, treatment methods and current potential uses: A review

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Abstract
About 20 per cent of the live weight of freshwater fish is visceral mass, a rich source of protein, lipids, polyunsaturated fatty acids, soluble vitamins, phospholipids etc having very great source of agro-industrial products. Any scientific technique that uses strong squanderers coming about because of fish harvesting by recuperating biomolecules like lipids and proteins brings about extra income as well as helps to reduce the dumping issues related therewith. Visceral fish hydrolysate (also known as fish protein hydrolysate; FPH) has drawn the interest of many researchers in recent years due to its great utility in the fields of food, pharmaceuticals, cosmetics and nutrition. The extraction or production of FPH including predefined sequential steps like collection of raw materials, pretreatment (alcohols, heat treatment, press technique coupled with heat treatment), hydrolysis (acidic, alkaline, enzymatic) and recovery of FPH using spray drying, lyophilisation, centrifugation or nanofiltration. The extracted FPH is characterized for molecular mass, protein content, structure of protein, IR, XRD etc to analyse the chemical nature of the FPH. It is widely used as emulsifier, binder, gelling agent, fertilizer, cryoprotectant and dietary additives in various industries. FPH has been used as potential source of microbial growth media for gram positive and negative microbes, nutritional supplement due to higher protein content (60-90%), and antioxidant, antihypertensive and antimicrobial agent. FPH is also used as nutriceuticals and some of the products are commercially available in various countries including US, Canada, UK. Considering the physicochemical difficulties associated with FPH, its excellent nutritional and functional properties generate possibilities for its usage in both the food and health industries.

Keywords: hydrolysates, fish viscera, antioxidant, antihypertensive, hydrolysis, purification

1. Introduction
India is the second largest manufacturer of freshwater farmed fish in the world. One of the most used sources of protein for human consumption is fish and aquatic products (New, 2000) [30]. During 2018, India's total fish production amounted to almost 6.24 million metric tones (MMT), which was very close to two-thirds of the total fish produced in the country from both sources of catch and cultivation (Lauria et al., 2018) [25]. Continuous increases in fish production, at an annual average rate of 3.2%, and an increase in fish intake per capita from 9.9 kg in 1960 to 19.2 kg in 2012 have resulted in to formation larger amounts of organic ravage (Freitas-Júnior et al., 2012) [13]. Scales, skin, visceral mass (viscera, air bladder, gonads, and other organs), head, and fins are among the by-products produced during fish processing. About 20 per cent of the live weight of freshwater fish is visceral mass and is a rich source of protein, lipids, polyunsaturated fatty acids, soluble vitamins, phospholipids etc having very great source of agro industrial products. About 91 million tonnes of fish are harvested per year, 29.5 percent of which is turned into fishmeal. Depending on the processing stage and fish species, production results in waste of 20-80 percent. Maybe more than 50 percent of the remaining fish tissue processes waste and is not consumed as food, causing burdensome issues with recycling and environmental concerns (Bhaskar et al., 2011) [5]. Any scientific technique that uses strong squanderers coming about because of fish harvesting by recuperating biomolecules like lipids and proteins brings about extra income as well as helps to reduce the dumping issues related therewith (Vidotti et al., 2003) [41]. Keeping all these things into consideration, scientific studies and research has been carried out to explore innovative and effective innovations that could reduce the burden of environmental challenges.
In all these studies a kind of protein called visceral fish hydrolysate (also known as fish protein hydrolysate) has drawn the interest of many researchers in recent years due to its great utility in the fields of food, pharmaceuticals, cosmetics and nutrition (Thiansilakul et al., 2007) [37]. Fish visceral hydrolysate is protein obtained from the visceral waste mass of fish including gut, liver, fins and other organs under specific temperature, pH and enzymatic conditions using pepsin, trypsin, alkalse and chymotrypsin etc (Chalamaiah et al., 2012) [7]. Fish protein hydrolysate (FPH) may be liquid or amorphous hygroscopic powder with nearly 81-93 percent protein, 3-8 percent ash, less than 5 percent fat and 1-8 percent humidity. The liquid FPH is a watery combination of hydrolyzed proteins with almost 90% moisture, which makes it particularly unstable for long-term storage and makes transportation difficult. Therefore dried FPH is preferred over liquid FPH due to greater stability, extended shelf life and ease in transportation. However, the removal of almost 90 percent of water from liquid FPH is the major challenge and quite costly for production of dried FPH (He et al., 2013; Silva et al., 2014) [18, 19, 35]. Table 1 shows the approximate protein content in various waste parts of the fish.

| Sr. No | Fish waste part | Protein % |
|--------|-----------------|-----------|
| 1      | Viscera         | 9-23      |
| 2      | Head            | 11-13     |
| 3      | Back bone       | 10-15     |
| 4      | Skin            | 8-12      |
| 5      | Milt            | 14-27     |
| 6      | Cut offs        | 12-22     |

The FPH is widely used in food, agriculture, pharmaceutical and, biotechnology industry as emulsifier, binder, gelling agent, fertilizer, crayoprotectant and dietary additives (Kristinsson et al., 2000) [23]. The objectives of this review is therefore to provide a light on the agro-industrial potential of fish waste as a source, especially viscrera, to extract native proteins and hydrolysate and to clarify their manufacturing process, their chemical structure and their functional and bioactive properties.

2. Extraction (Production) Of Fish Visceral Hydrolysates
The production of FPH consists of predefined sequential steps by step procedure for separation of fish visceral hydrolysates. The steps include pretreatment, hydrolysis and recovery of FPH (Petrova et al., 2018; Villamil et al., 2017) [33, 42]. The goal of the development of protein hydrolysates from fish viscrera is to solubilize the protein source by chemical or enzymatic methods to boost its biological and nutritional value in order to obtain products of high added value and commercial importance that shape different size peptides. In this section we will discuss the detailed extraction process of FPH.

2.1 Collection of raw materials and pretreatment
Fish and fish left-overs may be raw materials for the processing of FPH. Although the production can be influenced by the use of whole fish or just fish by-products. In order to ensure successful recovery of protein fraction, raw materials should be closely analyzed at the technical line. Production process of FPH starts with the collection of raw materials i.e. various fish parts including viscera, scales, fins and carcass etc. The collected fish left overs are cleaned and washed with water to remove the dirt, debris and other unwanted parts from the surface of tissues and then these are chopped in to smaller pieces (mincing) so as to facilitate the effective treatment and extraction procedure. The primary target of pre treatment is to obtain the concentrated protein from smaller pieces of the fish viscera. In addition, their aim is to prepare homogeneous water and chopped viscrera mixtures with the lowest fat percentage possible and other undesirable components for subsequent hydrolysis (He et al., 2013) [18, 19]. In order to produce a solvent (acid or alkali) or enzymatic hydrolysis, water, chemicals or enzymes are added to the minced raw material. There is a need to interrupt the hydrolysis process by chemical or thermal treatment according to the hydrolysis procedure after a certain time when a certain desirable degree of hydrolysis is reached (Petrova et al., 2018) [33]. In order to extract the attached fats from the fish tissues, heat treatment, the use of solvents and pressing methods have been commonly used. Heat treatment is generally known among all techniques because it satisfies both the purposes of fat reduction and inactivation of the enzyme (Guérand et al., 2001) [16, 17]. After heat treatment the solid residue is separated by using centrifugation technique. Similarly, (Valenzuela et al., 2001) has successfully used pressing technique coupled with heat treatment to get the solid mass followed by drying to obtain the moisture content less than 10 percentage (Valenzuela et al., 2001) alcohols such as ethanol, isopropyl alcohol, are also commonly used for the defatting in which fish minced are mixed with alcohols under continuous stirring for a fixed period of time (Dong et al., 2008) [41]. This approach is useful for minimizing the decay of bacteria as well as removing the fishy odor and bitter flavors (Hoyle et al., 1994) [29]. A modern technology has been used in some experiments to extract proteins from fish tissues. In addition to centrifugation and filtration, it consists of the solubilization of protein in acids or alkaline solutions to extract insoluble compounds; once separated; the proteins are precipitated by changing the pH to the isoelectric point and collected by centrifugation or decantation (Nolsøe et al., 2009) [31].

2.2 Hydrolysis of pretreated material
Three different methods can be used to prepare protein hydrolysates from fish processing discards, namely the acid hydrolysis, alkali hydrolysis, and enzymatic process. Below is the underlying process, effects of multiple variables and advantages and drawbacks of each technique. To extract free amino acids and low molecular weight peptides, protein hydrolysis carried out for cleavage of peptide bonds by absorbing a molecule of water for each broken bond. In aquaculture and fisheries, the hydrolysis mechanism (chemical or biochemical) has recently been used to produce more appropriate and viable goods (Pasupuleti et al., 2008) [32].

2.2.1 Acid hydrolysis
It is a low-cost, easy and simple process that makes it important at the industrial level. The complete fish protein hydrolysis using hydrochloric acid or, in some cases, sulfuric acid has been performed at elevated temperatures and also at high pressures. The solution is neutralised to pH 6.0 to 7.0 after hydrolysis, condensed and dried further. Absolute fish protein substrate hydrolysis can be accomplished in 18 hours at 118 °C in 6 M HCl. Due to the low cost of processing and the consequent widespread hydrolysis, this method is often
used to turn underused and secondary raw material from fish into fertilizer (Elavarasan et al., 2019) [12]. However during acid hydrolysis, important amino acids, such as tryptophan, methionine, cystine, are normally lost. In addition, asparagine and glutamine are converted into aspartic acid and, respectively, glutamic acid. In addition, owing to the formation of salts during the neutralization process, acquired hydrolysates have weak functional properties. Therefore, many methods of separation have been suggested, such as nano filtration and the use of ion exchange resins, with excellent performance (Pasupuleti et al., 2008) [32].

2.2.2 Alkaline hydrolysis
Wide water-soluble polypeptides are easily broken and further reduced at a slower pace into basic compounds in the alkaline hydrolysis phase of fish protein. Sodium hydroxide is mainly used in the alkali hydrolysis process. A high pH of 12.5 at 95 °C for 20 minutes was used in the small-scale batch system developed to hydrolyze the fish protein concentrates to increase functionality (Elavarasan et al., 2019) [12]. The key drawback of this process is the development of low amino acid content hydrolysates such as cystine, lysine, arginine, serine, threonine, isoleucine and residues such as lanthionine and lysinoalanine (Tavano et al., 2013) [36].

2.2.3 Enzymatic hydrolysis
In peptide bond cleavage, enzymatic hydrolysis uses mild conditions and is easier to control and more accurate; in addition, there are no side effects or losses in nutritional value, and it is easier to recover protein and purify those peptides (Tavano et al., 2013) [36]. Enzymatic hydrolysis is typically done from one to several hours at mild conditions; marginally elevated temperatures (typically about 35-65 °C) and a certain pH according to the optimal requirements of the enzymatic systems used; alkali (e.g. alkalase), neutral (e.g. papain, bromelain, alkalase, neutrase, flavourzyme) or acid (pepsin). Animal (pepsin), plant (papain,) bromelain) or microbial origin (alcalase, flavourzyme, neutrase) are enzymes used for the production of FPH. It is understood that microbial enzymes have greater pH and temperature tolerance (He et al., 2013) [18, 19]. It is also possible to produce protein hydrolysates by using proteases found in the digestive system of fish, such as pepsin, trypsin, chymotrypsin, collagenase and elastase (Vannabun et al., 2014) [39]. Due to many peptide bonds and their particular accessibility to enzymatic reactions, enzymatic hydrolysis of proteins is a complex operation. Enzyme specificity is not the only aspect that determines the final product's peptide profile and factors such as temperature and pH play an significant role. The temperature and pH may have a substantial influence on the kinetics of the enzyme reaction and their effect on each enzyme is different. Generally, when the enzyme is most active, there is an ideal balance of both pH and temperature. Enzymes are deactivated by temperature and pH extremes by denaturating them (Vázquez et al., 2004) [40]. Protein hydrolysates have been produced at autolysis temperatures ranging from 40-50 °C C and from the heads and viscera of sardinella Sardinellaaurita at pH 8, which showed that adding an additional enzyme will speed up the response and increase the degree of hydrolysis (Bougat ef et al., 2008) [40]. In spite of the above advantages, when enzymatic hydrolysis is used, there are some drawbacks. High prices of industrial enzymes, poor yields, the need for special care to deactivate enzymatic hydrolysis, the complexities of process management in achieving a certain molecular mass of the protein degradation products and the bitterness of the final FPH are among them (He et al., 2013) [18, 19].

2.3 Recovery of FPH
Depending on the final application of the hydrolysates, FPH can be recovered or purified by different processes including centrifugation, nano-filtration, ultrafiltration, microfiltration, and ion exchange chromatography. The most used of the above was centrifugation, from which four stages are obtained: oil fraction, emulsion sheet, FPH and sludge (Pasupuleti et al., 2008) [32]. It is dehydrated through lyophilization or spraydrying after separating FPH by decantation to increase shelf life and provide greater ease of handling, transportation and storage. A creamy white powder with enhanced functional and bioactive properties is usually the final product and it should be stored below 0°C to enhance the stability of the product (He et al., 2013) [18, 19].

3. Characterisation of FPH
The purified FPH is subjected to various characterization techniques in order to confirm the molecular nature and other characteristics of the FPH. The characterization of FPH is generally done for the estimation of protein content using Lowry’s method, molecular weight determination using polyacrylamide gel electrophoresis (SDS PAGE), protein structure using circular dichroism, detection of protease activity by Zymography, molecular mass detection by using orbitrap high resolution liquid chromatography mass spectrometry (O-HRLCMS), enzyme kinetic study using Lineweaver-Burk double reciprocal plot, detection of functional groups using infra red spectrophotometry and crystalline or amorphous nature of FPH can be determined by using X –ray powder diffraction study (Chaur et al., 2020; Geethanjali et al., 2013; Kameshwar et al., 2014; Mohanty, 2020) [9, 14, 22, 27].

4. Applications of Fish Visceral Hydrolysate
Fish protein hydrolysate (FPH) is fish oil containing a combination of amino acids and peptides of varying molecular sizes. In the food, cosmetics, pharmacy, nutriceuticals and practical food markets, FPH finds use. The Indian marine food industry provides a large amount of protein-rich waste that can be efficiently processed into fish protein hydrolysate by an optimized approach to the higher return of the seafood processor. In this section we will discuss various applications of FPH in detail.

4.1 FPH as potential source of microbial growth media
FPH obtained from Catla (Catla catla), Rohu (Labeo rohita), tuna and other species of fishes were found excellent growth promoters for various species of bacteria, yeasts and molds due to high content of peptides, amino acids and other essential ingredients (Guérard et al., 2001) [16, 17]. As a growth vector for various species of microorganisms (pseudomonas, vibrio and squib), fish viscera peptides from tuna, yellow stripe, swordfish, rainbow trout and squid were also analyzed which, because of their pathogenic or probiotic properties, are of concern to aquaculture (Vázquez et al., 2004; Husin et al., 2015) [40, 21] studied comparison of microbial growth on fish waste peptides from different hydrolysis methods. For peptide recovery, their research used mixtures of sardine and mackerel fish wastes. As nitrogen supplies for microbial growth, recovered peptides from various hydrolysis methods
(alkaline and enzymatic) were supplied. The efficiency of Peptone in microbial production was found to be impaired by the process of hydrolysis. Development of *Saccharomyces cerevisiae* using enzymatic peptone (Alcalase enzyme) was superior compared to development using other peptone forms. For Bacillus subtilis growth, the same pattern was observed. The method of hydrolysis played an important role in the development of peptone with a high protein content and degree of hydrolysis, which led to better microbial growth efficiency of the fish waste peptone. In conclusion, peptones derived from a sardine and mackerel waste mixture are theoretically viable to replace current commercial peptones as a microbial culture medium (Husin et al., 2015) [21]. Different peptones obtained from hydrolysed visceral homogenates of Bolti fish (*Tilapia nilotica*) residues demonstrated their suitability to promote the growth of lactic acid bacteria (mainly Lactobacillus sake Lb 706). The findings showed that the Bolti fish hydrolysatate provided impressive results in expensive commercial media, explicitly recommended for the cultivation and large-scale development of lactic acid bacteria (Deraz et al., 2011) [10]. FPH derived from cod stomachs demonstrated better efficiency for the development of pathogens such as *Vibrio anguillarum* and *Aeromonas salmonicida* than commercial products. (Gildberg et al., 2010) [15]. As a combined source of nitrogen, amino acids and vitamins in microbial growth media, three hydrolysates produced from cod viscera by various enzymatic hydrolysis procedures have been tested against *Escherichia coli*, *Bacillus subtilis*, *Lactobacillus sakei*, *Saccharomyces cerevisiae* and *Aspergillus niger*, the performance of these viscera hydrolysate was compared to the performance of typical commercial peptones. The findings suggest those fish hydrolysates are generally attractive alternatives to other forms of commercial nitrogen currently available (Aspno et al., 2005) [3]. As growth media for the bacterial strains of *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Streptococcus faecium*, *Listeria monocytogenes*, *Bacillus subtilis*, *Bacillus licheniformis* and *Micrococcus lysodeikticus*, tuna head hydrolysate, using the industrial enzymes Alcalase ® (Alc) and ProtamexTM (Prot), have been studied as an alternative to widely available complex sources (peptones and/or extracts) (Safari et al., 2012) [34]. These evidences clearly demonstrated the potential use of FPH as microbial growth promoter including both gram positive and negative bacteria. The type of hydrolysis, enzyme used and other conditions like temperature, pH are important factors to be considered during extraction of FPH and that governs the suitability and usefulness of FPH as microbial growth promoter. The extensive research work is still needed in this area.

### 4.2 FPH as nutritional supplement

FPH produced from the proteins of fish are excellent nutritional supplements can be easily used as bioactive compounds and absorbed and used for diverse metabolic operations. FPH can be used in multiple foods as usable additives because they have many major and special properties, such as water holding ability, oil absorbing capability, protein solubility, gelling activity, foaming ability and emulsification capability (Chalamaiah et al., 2010) [8]. In different hydrolysis conditions, the amino acid composition of FPH from different raw materials generated using different enzyme sources is predicted to vary. In general, the essential amino acids needed are abundant in FPH, with a rich content of glutamic and aspartic acid. FPH has non-essential amino acids, too. The presence of aromatic amino acids in hydrolysates of fish frame protein has been confirmed. Studies have clearly demonstrated that an optimal supply of important amino acids may be FPH from fish meat / fish waste (Elavarasan et al., 2019) [12]. The use FPH have been already explored in various food products including rice, snacks, fish and meat products, crackers and cereals but very limited number of clinical trials have been performed on FPH (Marchbank et al., 2008) [26]. The following table represents the approximate composition of some of the nutritional components present in FPH (Marchbank et al., 2008) [26].

| Sr. No | Nutritional component | % present |
|--------|-----------------------|-----------|
| 1      | Protein               | 60-90     |
| 2      | Moisture              | Less than 10 % |
| 3      | Fat                   | Less than 5 % |
| 4      | Ash                   | 0.45 o 27% |

### 4.3 FPH as bioactive agent

The potential of hydrolysates as antioxidants, antihypertensives and antimicrobials from several sources of fish protein has been studied. A peptide with high antioxidant properties was isolated from *Megalaspis cordyla*, a horse mackerel viscera protein. To extract possible protein hydrolysate, in vitro gastrointestinal digestion was used and subjected to sequential chromatographic methods utilizing quick protein solvent. The extracted protein had shown greater antioxidant property than natural alpha tocopherol in protection of polyunsaturated fatty acid (PUFA) (Kumar et al., 2011) [24]. Similarly, FPH derived from *Sardinella aurita* heads and viscera showed excellent scavenging effect on free radical DPPH and inhibition of linoleic acid. The findings of autoxidation show that the above peptides demonstrated more than 50 percent linoleic acid peroxidation inhibition and roughly 41 percent antioxidant function (Kumar et al., 2011) [24]. Five commercially available proteases, including alkali protease, papain, neutral protease, pepsin, and trypsin, were hydrolyzed with Pacific abalone (*Haliotis discus hannaino*) viscera, which are usually discarded as by-products. The hydrolysates obtained were fractionated using the bioreactor method of the ultrafiltration membrane. The resulting hydrolysate fractions, including the scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the scavenging effect on the hydroxyl radical, and the decreasing strength, were investigated for their antioxidant activities. The findings revealed that the hydrolysate fractions were efficient antioxidants and that the hydrolysates' antioxidant activity was related to the form of enzyme used. The abalone viscera may be used as a by-product as a possible source of antioxidant peptides (Zhou et al., 2012) [43]. Hydrolysates of the fish viscera protein obtained from *Parastromateus niger* have a protective effect on DNA against hydroxyl radical damage. The presence of antioxidant peptides in hydrolysates derived from fish co-products indicates both these findings (Nazeer et al., 2011) [29]. It has been well known that FPH peptides are rich source of arginine, valine, and leucine and other types of amino acids. Reports indicate higher antihypertensive behavior in peptides with low molecular weight (< 1 kDa) and shorter chain length (< 20 amino acids). Because these peptides have shown the inhibitory function of

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Angiotensin Converting Enzyme in vitro and in vivo, their ability to be used as antihypertensive drugs is outrageous. However, to provide an improved understanding of the interaction of peptides with the enzyme, the new emphasis on study in the field of molecular docking is important (Benjakul et al., 2014) [14, 39]. Angiotensin converting enzyme inhibitory characteristics of visceral hydrolysate proteins and peptides have been identified with (IC50 value 75µg / ml) and sardinella (IC50 value 0.8-1.2mg / ml), ribbon fish backbones (IC50 value 5.6µM) and yellow fin sole (IC50 value 28.7µg / ml), salmon heads, flathead, silver storage and barramundi (Abdelhedi et al., 2016) [1]. The angiotensin converting enzyme inhibitory (ACEI) activities of protein hydrolysates prepared from sardinella heads and viscera (Sardinella aurita) by treatment with separate proteases have also been investigated. The ACE inhibitory IC50 values for sardinelle protein hydrolysate by-products and fraction P4 were 1.2 ± 0.09 and 0.81 ± 0.013 mg / ml, respectively (Bougatef et al., 2008) [6]. Hydrolysis of the by-product proteins of cuttlefish with B alkaline proteases resulted in to the substance with excellent solubility over a wide pH spectrum and high ACE-inhibitory activity resulted from licheniformis. This research indicates that FPH may be used for the production of functional foods for hypertension prevention (Balti et al., 2010) [3]. Antimicrobial peptides are chains of amino acids that are less than 10 kDa by molecular weight and typically contain less than 50 amino acids, about all of which are hydrophobic. Against different Gram-negative and Gram-positive strains, nearly all antimicrobial peptides from fish have antibacterial behaviors. These antimicrobial peptides are possible candidates for modern medicinal antibiotic production as well as food industry antimicrobial agents. These antimicrobial peptides can also be used as antibacterial, antiviral, antimicrobial, immunomodulatory, and antitumor agents (Najafian et al., 2012) [28].

4.4 FPH as nutriceuticals

FPH has greater antioxidant, anti-obesity, anticancer, anticoagulant, immunomodulatory and antihypertensive activity so widely used in nutriceuticals. Table 3 describes the various commercial nutriceuticals products of FPH used in various countries.

5. Conclusion

FPH offers many valuable characteristics, making it desirable for its applicability in various sectors including agriculture, pharmaceuticals, biotechnology, food processing. The functionality, bioactivity, and nutritional benefits of FPH extend its scope of use. The physicochemical, functional and bioactive characteristics of the FPH and, subsequently, its use as a food component are prodigiously affected by the option of method used in the process of hydrolysis. Although considerable research has made strides, more more needs to be achieved. Considering the physicochemical difficulties associated with FPH, its excellent nutritional and functional properties generate possibilities for its usage in both the food and health industries.

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Table 3: Commercial nutriceuticals products of FPH marketed in different countries (Elavarasan et al., 2019) [12]

| Sr. No | Product | Details | Application | Country |
|-------|---------|---------|-------------|---------|
| 1     | Vasotenin | Bonito (Sardaorientalis) by thermolysin hydrolysis | maintenance of blood pressure | US and Japan |
| 2     | Amizate | Atlantic salmon fish proteins by autolysis | Sport nutritional product | North America |
| 3     | Protizen | enzymatic hydrolysis of white fish proteins | Dietary supplement to fight against stress and | UK |
| 4     | Seacure | Hydrolysis of deep oceanwhite fish proteins | Supports the cells of GIT And helps in functioning of Bowel | US and Canada |
| 5     | Molval | North Atlantic fish Molvamolva by enzymatic hydrolysis | For cholesterol regulation, stress control, for cardiac benefit | UK |
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