

EXTRACTION OF COLLAGEN THROUGH FISH WASTE FERMENTATION OPTIMIZATION AND ITS CYTOTOXIC STUDIES ON HaCaT CELL LINE

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ABSTRACT

More than 800 million tons of fish are utilizing in a year and 25-30% become waste. The waste amount is beneficial source for extraction of collagen. But procedure of extraction is still to be optimized. The current study was designed to extract collagen from fish through fish waste fermentation under various conditions. For collagen extraction we did lactic acid fermentation in which yogurt and Dough bacteria was added with fish sample and placed it in incubator at 30°C for one month. In yogurt and dough culture have at least 10 type of lactic acid bacterial species. After every week we check PH of each sample and take soup of that sample. After centrifugation of that sample's TCA (Trichloroacetic acid) precipitation was done of yogurt and dough sample and kept at -20°C. then did SDS-PAGE) using 6% resolving and 5% stacking gel of already stored sample. Then HaCaT cells (1×10^4 cells/well) were cultured in 96-well flat-bottom culture plates and treated with appropriate doses of FFCP (fermented fish collagen peptide) for 24 and 48 hours.

The SDS-PAGE revealed that the collagen protein of fish had doublet pattern for $\alpha 1$ and $\alpha 2$ chains at corresponding to 145 kDa and 132 kDa respectively. The density for $\alpha 1$ twice as compared to $\alpha 2$. Our result agrees that The fish collagen consists mostly of α -chain as well as little amount of

inter and intra molecular cross-linked components of α -chains; b (dimmer) and c (trimer). Different biochemical tests were done for identification of lactic acid bacteria catalase positive, citrate positive, urease negative. Our fermented fish collagen and peptides mixture were test for cellular cytotoxicity and proliferative effect on HaCaT cells. Current result shows that fermented extracted collagen are nontoxic and induce the proliferation of HaCaT cells. Current study is supported by various studies that revealed the medical application of fish extracted collagen as underline.

Keywords: Collagen, Environment, Fish Waste, Cytotoxic, Fermentation

LIST OF ABRIVIATIONS

TSE	Transmissible spongiform encephalopathy
BSE	Bovine spongiform encephalopathy
FFCP	Fermented fish collagen peptide
Ala	Alanine
Pro	proline
Gly	Glycine
CNS	Central nervous system
PrPsc	pathological prion protein
LAF	Lactic acid fermentation
LAB	Lactic acid Bacteria
DO	Dissolved Oxygen
HS	Hestrin-Schramm Media
DW	Distilled water
TCA	Trichloroacetic acid
SDS	Sodium dodecyl Sulphate
PAGE	poly acrylamide gel electrophoresis
TSI	Triple sugar Iron

1. INTRODUCTION

1.1: COLLAGEN

Collagen is the most abundant protein on earth and it contains 25-35% of the total body protein of animal's body. Collagen protein makes up the main constituent of bone and skin in animals and the collagen provide a glue as connective protein. In connective tissue of animal, the main structural protein is collagen. (Gelse *et al.*, 2003; Singh *et al.*, 2011). The recent era explored the use of collagen protein in various industries such as in pharmaceutical, biomedical, cosmetic, film and leather industries. (Gelse *et al.*, 2003; Ogawa *et al.*, 2004).The collagen protein is produced by plant, animal and fish at industrial level. But fish collagen protein provides several advantages over plant sources and animal sources protein (Subhan *et al.*, 2015).

The fish collagen is considered more valuable for use in biomedical as well as other health industries. In comparison of land animal collagen application, infectious diseases like transmissible spongiform encephalopathy (TSE), bovine spongiform encephalopathy (BSE) and mouth and foot diseases are not likely to be linked with fish collagen. Thus, wastes of fish are alternate source of collagen production (Jongjareonrak *et al.*, 2005; Nagai *et al.*, 2001).soluble form of collagens is beneficial in different fields like cosmetics, food and medicines due to its special features for example week antigenicity and biodegradability (Kołodziejska *et al.*, 1999). Due to its physical and chemical properties of fish collagen are completely change from animal collagen (Zhang *et al.*, 2007). Recently, only chemical and enzymatic method is used for extraction of fish collagen which are either might be toxic due to use of toxic chemicals for extraction or expensive due to use of expensive enzymes for extraction (da Rosa Zavareze *et al.*, 2010). Hence, the goals of this study are to find safe and cheap biological method for extraction of collagen protein from fish wastes and to characterize and apply in biomedical field such as skin care and protection.

1.2: EXTRACTION OF COLLAGEN

Chemical hydrolysis and enzymatic hydrolysis can be used for collagen obtaining (Zavareze *et al.*, 2009). Recently chemical extraction method for collagen is mostly used while in addition the use of enzymes is considered more nutritional but still there is requirement of an alternate procedure to extract collagen (Martins *et al.*, 2009). Additionally, less waste is produced by enzymatic

method and have short time interval but this process is very expensive. The chemical procedure comprises of the pretreatment use which is performed before the extraction of collagen may be extracted utilizing an acidic or basic process, that differs with respect to raw material origin. For the purpose of removing non collagenous substances and to get more yields pretreatment method can be utilized (Schmidt *et al.*, 2016). For the purpose of collagen extraction, the most commonly used methods are based on the solubility of collagen in neutral saline solutions, acidic solutions and acidic solutions with added enzymes (Schmidt *et al.*, 2016). Many known methods for extracting collagen, each method has its own limitations. Many efforts are focused to obtain collagen with high purity, high yield, reserved structural integrity and its unique properties including gel formation capabilities, water retention and thermal stability (Schmidt *et al.*, 2016). The acid base simplified method of collagen extraction.

1.2.1: Pre-Treatment:

Because of naturally cross-linking collagen in the connective tissues of animals' dissolves very slow, even in hot water. Small amount of chemical treatment is also needed to break crosslinks before extraction. (Schrieber and Gareis, 2007). In the end, the acids that is diluted and employed bases and collagen that is exposed to semi hydrolysis, that keeps the chains of collagen unbroken while crosslinks are cut into pieces. In pretreatment acidic form the waste materials are immersed in acidic solution till entry of solution by the substance. At a controlled temperature as the solution enters the skin structure it swells to two or three times of its actual volume and the breaking of the non-covalent inter and intra-molecular bonds occurs (Ledward, 2000). Acidic process is more suitable for more fragile raw materials with less intertwined collagen fibers, for example fish skins and porcine (Fernandes de Almeida *et al.*, 2012). This procedure is used to thicker substances which need most energetic entry through the use treatment agents, like, bovine ossein or shavings (Ledward, 2000). Sodium hydroxide and calcium hydroxide are commonly utilized for the purpose of pre-treatment, however sodium hydroxide is more suitable for pre-treatment of the skins because it causes significant swelling, which promotes collagen extraction by increasing exchange rate of the mass in the tissue matrix (Liu *et al.*, 2015).

1.2.2: Chemical hydrolysis

Organic acids like citric acid, lactic acid and acetic acid and inorganic acids like hydrochloric acid are used for acid hydrolysis. Though, organic acids have observed more effective compare to inorganic acids (Liu *et al.*, 2015; Skierka and Sadowska, 2007). The non-cross linked can be dissolve by organic acids and also the inter-strand cross-linked in collagen can be break by it; which the very high solubility of collagen at the time of extraction process (Liu *et al.*, 2015). Hence for the extraction of collagen acid solution, particularly acetic acid is used commonly. The pre-treatment materials has to be added to the acid solution in order to extract acid soluble collagen, those could be 0.5 M acetic acid and should be hold under continuously stirring at 4C° for 24-72 hours depending on the type of raw material (Kaewdang *et al.*, 2014; Liu *et al.*, 2015; Nagai *et al.*, 2000).

Filtering process is needed to isolate supernatant from the collagen after the extraction process, which has state of liquid. After that the filtrate is precipitated with NaCl to get the collagen powder. Centrifugation has to be used to collect the precipitate and later on it could be re-dissolve in minimum volume of 0.5M acetic acid and then dialyzed in 0.1 acetic acid for 48 hours, and then distilled water are applied for 2 days, with replacement of the solution on average every 12 hours (Schmidt *et al.*, 2016).

1.2.3: Enzymatic hydrolysis:

There is strong intermolecular covalent bond in telopeptide region of triple helix structure in collagen, which cannot be sliced by using only acetic acid which specifies enzymes like pepsin from fishes, mammalian, trypsin, papain, alkaline protease, bromelain collagenase etc. which is added to remove non-helical structures by increasing collagen solubility. Out of all these, most utilized enzyme for marine collagen extraction is pepsin (Ehrlich *et al.*, 2010; Silva *et al.*, 2014). The triple helix structure is maintained by whole isolated collagen, specially collagen type I having same shape and amino acids arrangement.

The triple helix structure maintains in all extracted collagen, as the amino acid profile and similar morphology of type I collagen. Chemical analysis shows less points of interest as compared to spectral analysis of enzymatic analysis, e.g. specificity, degree control of hydrolysis, low level of

actions, and in last hydrolysate lower salt contents. Additionally, enzymes have very less use and there is no need to remove these enzymes from medium (Zavareze *et al.*, 2009).

1.3: CONSTITUENTS OF COLLAGEN

Main protein present in skin and other connective tissue, which is present in different forms in all tissues of multicellular organisms. It performs different roles based on its position (Schmidt *et al.*, 2016). From different species of animals, we can extract collagen and it is commonly from meat by-products. Bones, skin, tendons, and cartilage are important sources of collagen. Several other studies suggest that collagen can also be extracted from sources other than animals like birds and fish as substitute to bovine collagen from that has risk of bovine spongiform encephalopathy (Kaewdang *et al.*, 2014; Wang *et al.*, 2014).

The sharing of structure, composition and molar mass, and the consequent active structures and characteristics of collagen, rely on processing situations of that substances from which it is taken and the enzyme specificity used in the process of extraction. (Schmidt *et al.*, 2016). Therefore, it's important to keep in limit process of extraction for every raw substance to get good reward and high collagen characteristics for the preferred use. Collagen can be used as industrial application because it is one of the best biomaterial due to its broad variety (Schmidt *et al.*, 2016). In food industry collagen and gelatin has very high demand due to high content of protein and their handy stuffs, like gel formation, formation of stabilize emulsion ability and water absorption capacity. Collagen can be used as transporter for drugs, genes and proteins and also used for human skin, blood vessels and ligaments as substitute (Gómez-Guillén *et al.*, 2011; Kim and Mendis, 2006).

Collagen protein is present in every multicellular animals (Schmidt *et al.*, 2016). In vertebrates and invertebrates' collagen is an essential factor in the support structures. In mammals' collagen found most abundantly resulting of about 25% of the total weight of all protein (Badii and Howell, 2006; Schmidt *et al.*, 2016), and it is the essential protein of cartilage, bones, tendons tissues and skin particularly. Collagen in fishes and poultry play the same role as an invertebrates and is the essential constituent of the body wall (Badii and Howell, 2006).

In mammals have many type of collagen which contain typically of about 11% alanine (Ala) 21% proline (Pro), 35% glycine (Gly) and hydroxyproline. In collagen the amino acid sequence is usually repetitive triple peptide unit (Gly-X-Y), in which X is mostly Pro and Y is mostly Hyp

(Schmidt *et al.*, 2016). Until now about twenty-nine (29) type of collagen have been stated, which are placed based on their structures (Schmidt *et al.*, 2016).

1.4: STRUCTURE OF COLLAGEN

Members of every collagen family have same right-handed triple helix which have characteristics of three alpha chain as shown in figure 1 (Lethias *et al.*, 2010) The three identical polypeptide chain (homotrimers) may be creates the right handed triple helix structurally, as in collagen type (II, III, VII, VIII, X) and many others. Though mostly rich collagen types like (I, IV, V, VI, IX, and XI) are heteromers, which are right handed triple helix formed by two or more different heteromeric chains. An extensive left-handed helix which is created by each three α -chain and has a pitch of eighteen (18) amino acids per turn (Fallas *et al.*, 2009). The right-handed super helix which are formed by three α -chain have one staggered residue arranged and adjacent chain as a common central axis in a right-handed style around by supercoil to make the triple helix structure. The glycine characterizes the triple helical collagenous domain in every 3rd point of polypeptide chain which make a (Gly-X-Y)_n duplicate structure. The alpha chain assembled around central point in a way that all glycine residue is positioned in the center of the triple helix, while other amino acids larger chains are located in the outer side. This allowed the peptide chain fold strongly down the central point of the molecule while the steric clashes in the center of the assembly are been avoiding.

The proline and hydroxyproline frequently filled the X and Y positions. Depend on collagen type, some lysine and proline residue are altered by post translational enzymatic hydroxylation. For the development of intramolecular hydrogen bonds, the 4-hydroxyproline contents are vital which also helps in the conformation's stability of the triple helical. Between different types of collagen, the length of triple helical parts is different (Fallas *et al.*, 2009; Gelse *et al.*, 2003; Gordon and Hahn, 2010). In fibril forming collagen (I, II, III), with exemption of collagen type XXIV and XXVII, the (Gly-X-Y) replicate in the helix is the main three-layered helical domain. The length of fibril forming collagen are about 1000 amino acids residues and have perfect structure of Gly-X-Y triplet. For non-fibril forming collagen, in the triple helices have at least one interruption (Gordon and Hahn, 2010). N-propetide ore N-peptide is the fibrillar collagen end amino acids. Normally it

is consisting of about one small triple helical domain called minor helix.

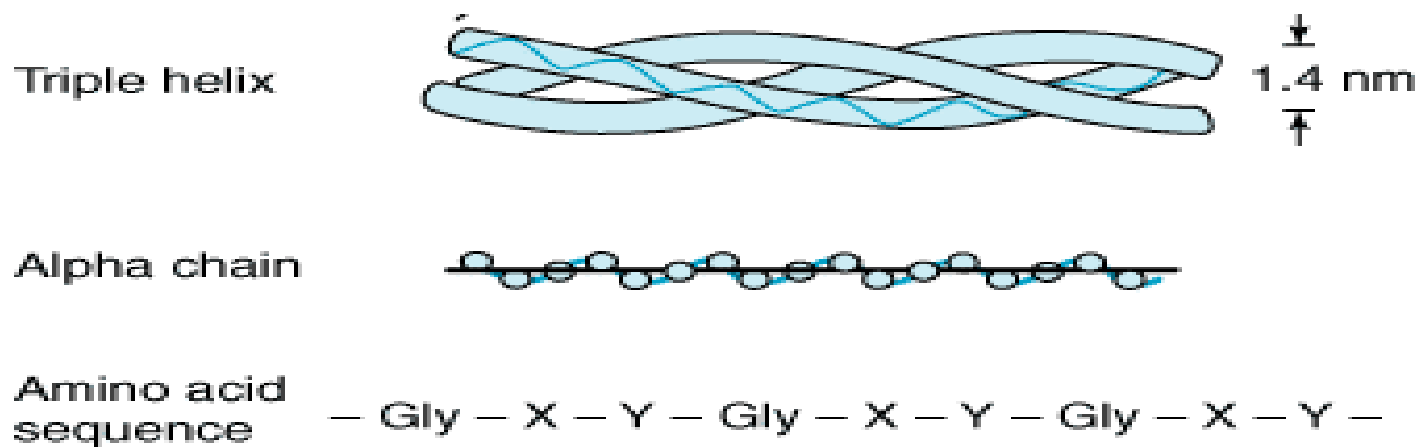


Figure 1.0-1: Molecular structure of a collagen triple helix in type I collagen molecule (Chhabra, 2013)

After the formation of major triple helix, the carboxyl and amino ends are processed. The processed molecule is ranged in a procedure of a quarter stagger in the increasing fibril. Fibrils of type I and type II collagens nucleate respectively by type V and XI collagens (Gordon and Hahn, 2010; Kadler *et al.*, 2008). The N-peptide of collagen V and XI regulate the fibril diameter which are retained after processing (Birk, 2001; Gordon and Hahn, 2010).

1.5: COMPOSITION OF COLLAGEN

Collagen peptides of fish have particular composition of amino acids with great content of proline, hydroxyproline and glycine. By ingestion of fish collagen, hydroxyproline peptides are not fully absorbed to free amino acid and in blood it can be detected. In the skin, bones and joints these hydroxyproline stimulate the cells and synthesis collagen by cell growth and activation (Subhan *et al.*, 2015). On age collagen type III intensely depends; mostly young age skin has about 50% collagen type III but as skin but with age collagen type III percentage can be fallen from 5 to 10%. In animal kingdom there are about twenty (20) different type of collagen. Every mature collagen consists of three polypeptide chain called α chain that might be different or same and are arranged in triple helical structure (Bella 2016). Every α chain can be identified by its nomenclature: $\alpha_n(N)_p$, n is the identification number of α chain, p is the number of polypeptide and N is the number of collagen.

Among organs collagen distribution are different and with type the length of collagen varies. Each polypeptide is 300 nm long (corresponding to about 1000 AA residues) in type I, II, III collagens (Burgeson and Morris, 1987; CHU *et al.*, 1987). E.g. in type I collagen each of the two identical $\alpha 1(I)$ chain contain 1056 AA residues. Collagen molecules pack together side by side in fibrous collagen through an interchain cross-link between allysine residues formed by the extracellular lysyl oxidase (Bella, 2016).

1.6: SOURCES OF COLLAGEN

The main industrial sources for collagen production are Bovine and porcine skins. In 1930, porcine skin was the first raw material used for collagen production and nowadays porcine skin is still the most significant raw material for large scale industrial production of collagen (Gómez-Guillén *et al.*, 2011). It has been reported that collagen of porcine skin has bovine spongiform encephalopathy (BSE), while collagen of fish has such chances of pathogen like BSE(Yamaguchi, 2002). Other than this, Jews and Muslims not accepting any food products related to pigs and Hindus not accepting or consume food products related to cow(Karim and Bhat, 2009b).

By- product from the slaughter of pork and beef are the key sources for collagen extraction (Ferreira da Silva and Barretto Penna, 2012; Jia *et al.*, 2010). Different by products already have been studied including tendon and the Achilles (Li *et al.*, 2009), bovine bones (Paschalis *et al.*, 2001), pericardium(Santos *et al.*, 2013) and inner layer of bovine skin(de Moraes and Cunha, 2013), porcine lung(Lin *et al.*, 2011) and porcine skin (Yang and Shu, 2014). Nowadays it is examined that by product of fish is alternate source for extraction of collagen (Kaewdang *et al.*, 2014; Mahboob, 2015; Muralidharan *et al.*, 2013; Ninan *et al.*, 2014; Tang *et al.*, 2015; Wang *et al.*, 2014). Bovine spongiform encephalopathy (BSE) belongs to family of disease known as transmissible spongiform encephalopathies (TSE), which affected by the increasing of pathological prion protein (PrPSc) in central nervous system (CNS) and brain that affect bovines of adult.

Thus, many researches have keen interest to find an alternative collagen source. For now, collagen of fish waste was found to have the most relevant features to collagen of mammals. Hence, it's important to draw the industry's attention to marine collagen(Kim and Mendis, 2006; Nagai and Suzuki, 2000).

therefore, many types of species of fish were examined as alternate source of collagen. Studies of all fish showed different denaturation temperature features. Due to this interest among research communities had raised in optimizing the yields and extraction condition and also characterizing the final collagen. These collagens were generally extracted from fish bone, scales and skin residues. Alaska Pollock, Atlantic salmon and Cod are living in cold water and were researched on their functional and physio-chemical properties. Species living in cold water showed significantly low denaturation temperatures (around 4-17 °C) while tropical species denaturation temperature is (around 18-29 °C). sub-tropical species were red Tilapia, channel catfish, yellowfin Tina, skate or grass carp, Nile perch (Gómez-Guillén *et al.*, 2011; Karim and Bhat, 2009b).

In this type of situation collagen of fish are best as it has no risk of transmission of disease, no religious barriers and have high availability. The key difference between animal collagen and fish collagen are high essential amino acids, high biological value and low concentration of hydroxyproline. the fish fins that is waste products of canned tuna processing are high quality collagen source extracted but with low yields (Aewsiri *et al.*, 2008)., Such as bigeye snapper(Kittiphattanabawon *et al.*, 2005; Nalinanon *et al.*, 2007), carp(Duan *et al.*, 2009), threadfin bream(Nalinanon *et al.*, 2008), yellowfin tuna(Woo *et al.*, 2008) etc. have been characterized and extracted.

Table 1-1: Comparative study between marine and mammalian collagen

S. No	Marine	Mammalian
01	Low melting point	High melting point
02	Cheap	Expensive
03	Easily available (high amount)	Difficult extraction (less amount)
04	Low viscosity solution	High viscosity solution
05	No risk of transmitted disease	High risk of transmitted disease
06	High contents of GLX and ALA with low PRO	Low contents of GLX and ALA with high PRO
07	Soluble in water	Soluble in organic solvent

Therefore, more importance has been given to alternate sources of collagen especially from fish bone, skin and scale (Nalinanon *et al.*, 2007; Skierka and Sadowska, 2007; Wang *et al.*, 2007). Fish offal like bones, skin, fins and scales has gain more importance as alternate source of collagen(Gómez-Guillén *et al.*, 2002). From the discarded portion of fish wastes fish collagen can be produced like fins, skin and scales which are rich sources of collagen(Dun *et al.*, 2008).

1.7: BIOFERMENTATION

Bio fermentation is a natural procedure that inevitably disturb the food supply of human around the world is fermentation. The word fermentation is a Latin word which mean fervere means to boil, which indicate the presence of yeast action on extract of salted grains or fruits during alcoholic beverages production. For microbiologist fermentation word mean any process which are used for production of different products of mass culture of microorganisms. On the other hand, for a biochemist, fermentation means a process which generating energy and organic compound reacts as both electron donor and acceptor. Fermentation occur in absence of oxygen in which

energy are produced. (Stanbury *et al.*, 2013). Foods which are produce after the action of microorganism or enzymes are fermented foods due to which significant modification of food and desirable biochemical changes occur.(Campbell-Platt, 1987). Fermentation technology plays an important role in technology of food. Fermented food is accepted throughout the world and rise in the human relation to the microbial world. Fermented foods like beer, wine, bread and yoghurt are mostly used as a food. Microbial ecosystem of fermented foods, like cheese, bread, yoghurt, beer and other foods constitute microbial ecosystem that present in human families throughout the world. Fermented food production doesn't require information of the biologically mediated nature of fermentation, because the biota that do fermentation are present throughout the world, either humans use them or not. The relationship associated with safe foods can be treated as microbial ecosystem, sat at the top of the counter and stored in food sales worldwide. The main ingredients of fermentation ecosystem include: (molds, bacteria and yeast), biological materials which are to be fermented, fermentation solution, a pot developed with a close door and different other resources that is developed can be used to be and fermentation monitoring. Consumed foods are protected by microbes that live in, food storage vessels (Scott and Sullivan, 2008).

Dried foods are counted as all our foods, which are mainly used for meat, milk, soybean, fish and plant products for commercial production. Acute food production is highest in Europe, Africa and North America. America in the south, large amounts of drinks and products of milk are fermented. fermented dairy products in the middle east are important and in the Indian subcontinent grain are fingers in high amount. In Southeast and Eastern Asia, grapes and fish are the most importantly consumed nutrition, both in the source of protein. In these areas, as well as the use of cereals, can be cumulative with fingers that produce soybean in soya and rice or yoghurt production with soybean in wheat. While many yeast food items are used where they are made, there are quantities in alcohol trade, spirits and wine through growing things, and for soy sauce and for fault (Campbell-Platt, 1994).

1.8: LACTIC ACID FERMENTATION

Lactic acid bacteria are non-spore forming or coconut, Gram-positive, growing, catalase negative lacking a hem source, generally sessile and rarely nitrate reducer. Glucose has used by them for fermentation and might be also homofermentative, about 85% lactic acid could be produce by

them from glucose or heterofermentive generating CO₂, ethanol and acetic acid or lactic acid in the equimolar volume from lactic acid (Dworkin, 2006). Genera lactobacillus strains lactococcus, enterococcus, Aerococcus, Tetragenococcus, Vagococcus, Camobacterium, Pisciglobus created L-lactic acid; several lactobacillus strains and Leuconostoc created D-lactic acid whereas many other lactobacillus, pedicoccus and Weissella strains created DL-lactic acid (König and Fröhlich, 2017; Tanasupawat *et al.*, 2011).

1.8.1: Condition for lactic acid bacteria (LAB) fermentation:

The most suitable environment for lactic acid bacteria (LAB) are lower ethanol contents, lower optimum PH, optimum temperature, and enough fermentable sugar contents. Furthermore, both LAB and yeast are capable to survive under less dissolved oxygen (DO) things due to which condition over turns a growth of aerobic general bacteria (Inoue *et al.*, 2013)

1.9: FISH FERMENTATION

The products of marine finfish and fresh water, shellfish and crustaceans can be denoted by fermented fish products which are results due to the joint action of fish enzyme and bacterial enzyme with salt to carry out fermentation and avoid putrefaction (Ruddle and Ishige, 2010; Saisithi, 1994). Textural change produced by the enzymes and helped in flavor production whereas bacteria involved flavor for fish fermentation and developed the aroma (Beddows, 1998). Area which has seasonal plenty of local fish, preservation of fish occurs in those area. Fish species of fresh water certainly found in native hydrological systems have been fermented. First time the process of fermentation was developed amongst sitting farmers and anywhere salt is obtained simply. Fermentation of fish has advanced in local regions of southeast and continental East Asia (Ruddle and Ishige, 2010). In East Asia a widespread range of fermented- fish has created that an exacting grouping by product variety should be restricted to separate states. Method of preparation and end product is key cause for its classification.

fish biomass Effective utilization (fish waste and by-catch fish) is vital. Biomass of fish has recognized rich in different nutritional elements like lipids, minerals and proteins (Council, 2002). Adding with this biomass of fish can easily ruin due to high nutritional content and high moisture. Generally, biomass of fish is preventing from spoil by heat drying and allowed it for utilization. About fish silage production many reports available (Espe and Haaland, 1992; Raa *et al.*, 1982).

Lactic acid bacteria which are usually found in raw material is used for the biological fermentation of fish waste. It could be carried out by chemical acidification using organic or inorganic acid, or introduced as starter culture (Raa *et al.*, 1982).

Fish fermentation wastes by means of starter cultures with mixture of Lactic acid bacteria (LAB) and yeasts was observed by Faid *et al.* By biological fermentation fish wastes is processed using a combine starter cultures of *Saccharomyces* species. and *Lactobacillus plantarum* to change wastes of fish into stable feed ingredient (Faid *et al.*, 1997). Process of producing of starter of sake (moto), soy sauce, Lactic acid bacteria help to avoid contamination of other bacteria because of a symbiotic relation with koji mold (*Aspergillus Niger*) and yeast (*Saccharomyces cerevisiae*) under lower acidity, allowing glucose produced by koji mold. Though in the later stage of the process LAB is known to die due to too much ethanol and acidity and high temperature by fermentation (Inoue *et al.*, 2013).

1.10: PROTEIN ANALYSIS

1.10.1: General consideration and classification of protein

protein is one of the abundant component of all cells, and about all excluding storage proteins are essential for cell structure and biological functions. Protein present in food are very complex. A high number of protein have been characterized and purified. Protein have different molecular mass, of about 5,000 to more than a million Daltons. Protein are consisting of different elements like carbon, hydrogen, oxygen, Sulphur and nitrogen. Building blocks of proteins are 20 α -amino acids. In proteins amino acids are linked by peptide bonds. The most distinguishing element present in protein is Nitrogen. But in various food proteins nitrogen content ranges from 13.4 to 19.1% (1) Because of amino acid variation of specific amino acids composition of proteins. Usually basic amino acids rich protein contains more nitrogen.

1.10.2: Classification of protein

Protein are classified by their structure, composition, biological function, or solubility properties. E.g. simple protein only consists of amino acids upon hydrolysis, but protein which are conjugated also consist of non-amino acid components. Protein has unique structure that can be change by heat, alkali, acid, 6 M guanidine-HCL, 8 M urea, detergents and organic solvents. Functional

properties as well as solubility of protein can be changed by denaturants. Protein analysis is complex because some foods components contains similar physical and chemical properties. Nitrogen which are non-protein could come from amino acids, nucleic acids, peptides, amino sugars, porphyrin, phospholipids, and some vitamins, uric acid, urea, ammonium ions, and alkaloids. That's why organic nitrogen in foods would signify nitrogen initially from proteins and have low extent from all other organic nitrogen which contain non-protein substances. On methodology dependent other main food components, containing carbohydrates and lipids can affect physically with analysis of food protein.

1.11: OBJECTIVES

- The study will focus to identify the bacteria involved in fish waste fermentation, and the optimization of identified bacteria for production and purification of Type-I collagen.
- To investigate the cytotoxic effect of collagen on HaCaT culture cell line.

2. MATERIALS AND METHODS

2.1: Study Area

The current study was designed from July 2017 to August 2018 at Department of Microbiology, Hazara university, Mansehra, KP, Pakistan.

2.2: Study purpose:

Study purpose was to do fermentation of fish through lactic acid bacteria for collagen type I extraction.

2.3: Specimens Collection and Storage

Siran River is one of the best fishing site and marketing place in District Mansehra that is situated near Hazara University. Fresh fish were collected from the Siran Fishing market in sterile bags and transported instantly to central laboratory at Department of Microbiology, Hazara University Mansehra. The specimens were washed three times and then kept at 4°C for 24 hours for further processing.

2.4: Preparation of HS Media (Hestrin-Schramm Media)

Ingredients

- 20g D-glucose
- 5g Peptone
- 5g Yeast Extract
- 2.7g Na₂HPO₄
- 1.15g Citric Acid
- 1000ml Distilled Water
- pH 6.0

Preparation of Media

- Vacuum Electronic Balance was used for the measurement of ingredients.
- The ingredients were mixed with distilled water by stirring.
- Then autoclaved at 121°C for 15 minutes.
- After cooling (45 °C-60 °C), 2.5ml of antifungal (Nystatin) was added to the media.
- The media was preserved on room temperature at appropriate environment until further use.

2.5: Preparation of Agar Plates:

- 100 ml of already prepared HS media was poured into flask
- 6 g of agar was added into the media
- The media was heated on heat stirrer up to gently mixing of agar with media
- The media was remained to cool to be handled easily
- The media was divided into equal concentrations on 10 different sterilized petri plates
- The petri plates were stored at 4 °C.

2.5.1: Culturing on Petri Plates

- The petri plates were transferred to laminar flow unite.
- Dry dough was diluted in distilled water.
- The diluted dough was streaked on two different petri plates containing HS agar media through sterile swab.
- Similarly, yogurt was also streaked on two different HS agar petri plates.
- The plates were then incubated for 24 hours at 30 °C.

2.6: Specimen Processing

The fish specimens were cut into 1-2 cm pieces and put into three different fermentation experimental media and a control medium in four different flasks (flask 1-4). Flask 1 contained HS media along with Dough culture, flask 2 contained HS media and Yogurt, flask 3 contained only HS media and flask 4 contained only distilled water (control medium). The flasks were then placed in incubator at 37 °C. The samples (soup specimens) were taken on weekly bases from the incubated flasks.

2.7: Extraction of Type 1 Collagen

2.7.1: Trichloroacetic acid (TCA) Precipitation

10ml of soup specimen was taken in four different 50ml tubes from the flasks on weekly bases e.g. day 0, day 7, day 14, day 21 and day 28. One each week, the pH of the flasks was observed and noted carefully. The specimen was centrifuged on 4 °C at 4500 rpm for 10 minutes on ultra-centrifuge. After ultra-centrifugation, 3ml of soup was taken and transferred in to 15ml tubes. Then these tubes were centrifuged at 2000 rpm for 5 minutes on ordinary centrifuge. Then from each tube, 900µl of soup was transferred to 12 different 1.5ml Eppendorf tubes (3 tubes of each sample) and 100 µl of TCA was added to each tube and mixed constantly. The Eppendorf tubes were then kept on -20 °C for 20 minutes.

After 20 minutes of freezing, the samples were centrifuged at 12,000 rpm for 10 minutes on Microspin. After centrifugation, the soup was discarded and 1ml of 80% cold Acetone was added to each tube and centrifuged again at 10,000 rpm for 3 minutes on Microspin. The Acetone was discarded after centrifugation and 1ml of fresh Acetone was added and centrifuged again. This process was repeated 2-3 times. After centrifugation, the Eppendorf tubes were put up side down for drying. After drying, 100 µl of distilled water was added and preserved at -20 °C for further processing.

2.7.2: Gel Electrophoresis**6% Resolving Gel SDS-PAGE****Requirements****Components Gel Volume (10ml)**

• 30% Acrylamide mix	2 ml
• 1.5 M Tris (pH 8.8)	2.5 ml
• 10% SDS	0.1 ml
• 10% Ammonium Persulfate	0.1 ml
• TEMED	0.008 ml
• Distilled water	5.3 ml

5% Stacking Gel SDS-PAGE**Requirements****Component Gel Volume (5ml)**

• 30% Acrylamide mix	0.83 ml
• 1.0 M Tris (pH 6.8)	0.63 ml
• 10% SDS	0.05 ml
• 10% Ammonium Persulfate	0.05 ml
• TEMED	0.005 ml
• Distilled Water	3.4 ml

Methodology

The collagen type 1 was hydrolyzed by SDS-PAGE (sodium-dodecyl sulfate polyacrylamide gel electrophoresis) with 6% resolving gel. The gel was then kept for 45 minutes. The gel was pipetted with isopropanol to avoid oxygen inhibition of the polymerization reaction and to make the surface uniform. Then the 5% stacking gel was loaded over the resolving gel and kept for 30 minutes. Prior to electrophoresis, 100 μ l of SDS buffer (denaturing buffer) was loaded in to it and heat shock of 95 °C for 5 minutes was given to the samples to convert the complex protein structure to linear form. Then Gyangnam protein marker and 10 μ l of each sample was loaded on gel wells. The gel tray was placed in Glycine buffer and run for 25 minutes of at 100 volts. After 25 minutes, the electric voltage was increased to 200 volts to achieve the perfect bands on resolving gel. Once the electrophoresis was completed, the gel was dyed with Coomassie Blue Brilliant G-250 Dye and incubated in shaking incubator for 24 hours at 30 °C. then the samples were washed with acetic acid and methanol and photographed subsequently. Collagen type 1 and its hydrolyzed form were observed clearly.

2.8: Cell Culture and Reagents

HaCaT cells, spontaneously immortalized human epidermal keratinocytes, were purchased from CLS Cell Line Service and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin in a humidified 5% atmosphere at 37°C. Sub-confluent cells were harvested with trypsin-EDTA and used for further experiments.

2.8.1: Cell cytotoxicity Assay

HaCaT cells (1×10^4 cells/well) were cultured in 96-well flat-bottom culture plates and treated with appropriate doses of FFCP for 24 and 48 hours. Cell viability was determined using the colorimetric WST-1 conversion assay using EZ-Cytox assay kit and as per manufacturer's instructions and the percent cell viability was calculated. Experiments were performed in triplicates.

2.9: Biochemical tests for identification of Bacterial species used in fermentation

Bacterial culture is commonly identified by different biochemical tests. These biochemical tests are applied for the identification of enzymes, proteins or peptides based on biochemical activities of these enzymes and peptides. These biochemical tests give an accurate result in short time. Some biochemical tests are used in this study to identify the bacteria. Some biochemical tests used here are; oxidase test, catalase test and urease test.

2.9.1: Catalase test

This test is commonly used for the identification of an enzyme catalase secreted by many bacterial species during the growth. Some of the bacterial species produce this enzyme to catalase hydrogen peroxide to oxygen and water molecule. *Bacillus* species produce catalase enzyme which convert H_2O_2 to oxygen and water molecule. When bubbles are produced in the solution, then the bacterial sample is indicated as catalase positive.

Principle

The enzyme converts hydrogen peroxide to oxygen and water molecule. Bacterial culture to be tested is brought in contact with the hydrogen peroxide in a sterile test tube to check out the catalase activity of the culture. Whenever a bacterial culture is catalase positive it produces oxygen in the form of bubbles in the test tube. Which indicates that the culture is catalase positive. But one thing should be noticed that the culture to be tested should be fresh and should be 24 hours old.

Reagents

Hydrogen peroxide (3%), is used for the identification of catalase enzyme.

Procedure

About 2-3 drops of H_2O_2 is poured into an autoclaved test tube. 2-4 similar culture colonies are picked through an autoclaved cotton stick and are mixed with the solution present in the test tube. The catalase positive bacterial culture releases an enzyme, catalase enzyme, which converts hydrogen peroxide to water molecule and oxygen in the form of bubbles. The bubbles are examined by naked eyes within a few seconds.

2.9.2: Urease test

Some bacterial species hydrolyze urea and convert it into constituents within a short time, like bacillus species. This test is used routinely as a diagnostic laboratory mostly.

Principle

Some bacterial culture produces an enzyme which may be able to hydrolyze urea into carbon dioxide and ammonia in the presence of water molecules. The combination of these sub constituents, water molecules, ammonia and carbon dioxide results into ammonium carbonate. The presence of ammonium carbonate turns the culture medium into alkaline and turns the color of phenol red (an indicator) from yellow to bright pink color.

Procedure

The medium is prepared and autoclaved at $121^\circ C$ for 15 minutes. After, the medium is filled into the autoclaved test tubes. The broth medium in the test tube is inoculated with the help of a sterilized wire loop. Pure culture is inoculated into the test tubes. The slant of each test tube is inoculated with the sample culture. After, the caps of the test tubes were kept at loose condition and the samples were incubated at $35^\circ C$ for about 20-24 hours. When the color of the medium turns to pink color, means alkaline condition.

2.9.3: TSI (triple sugar iron):

It is a microbiological test to test capability of microorganism's ability that can ferment sugar and can produce H_2S (hydrogen sulfide). It is a test that has triple sugar (glucose, sucrose, lactose) and iron.

Composition of TSI:

Glucose, lactose and sucrose at concentration of 1:10:10(1-part glucose, 10-part lactose and 10-part sucrose).

Phenol red: for identification of acidification (it is red in alkaline condition and in acidic collection it is yellow).

It also has peptone as a source of nitrogen because in aerobic condition peptone when utilize it produce ammonia.

Iron: indicator of hydrogen sulfide.

Procedure:

1. bacterial colony was picked with a well sterilized wire lope.
2. Inoculate TSI agar in the test tube and made slants of TSI medium.
3. the cap was covered loosely and incubate it at 35°C for 24 hours.

2.9.4: Indole test

A test performed to observe the trait of an organism which can use and split tryptophan amino acid and form the indole compound.

An enzyme tryptophanase hydrolyzed tryptophan to create three possible final products, indole is one of them. Kovac's or Ehrlich's reagent are comprised of 4(p)-dimethylamino benzaldehyde, which react with indole and produce red color compound which is a sign of indole production.

Two methods are in used;

- A test detect the very active creating indole organism called spot indole test.
- Passive creating indole organism are detected by conventional tube method which need require overnight incubation.

Methods

Broth culture was inoculated to the tryptophan broth or the isolated colony of tested organism was emulsifying in tryptophan broth. Then it is incubated at 37°C for 24-28 hours, and then 0.5ml of kovac's reagent was added to the broth culture.

2.9.5: Citrate test:

The citrate agar is used to check the ability of microorganisms that can use citrate as a sole of energy. Medium contain $(\text{NH}_4)_2\text{PO}_4$ (inorganic ammonium salt) as a source of nitrogen and citrate as carbon source. Bacteria which can grow in citrate agar media produce an enzyme called citrate permease which has the ability to convert citrate to pyruvate.

Composition:

Table 2-0-1: Composition of citrate agar medium

Agar	15.0 gm
Dipotassium phosphate	1.0 gm
Ammonium dihydrogen phosphate	1.0 gm
Magnesium sulfate	0.2 gm
Bromothymol Blue	0.08 gm
Sodium chloride	5.0 gm
Sodium citrate	2.0 gm
Deionized water	1000 ml

Procedure:

1. Well isolated Bacterial colony was picked and streak the citrate slant back and forth.
2. Placed that citrate slants in incubator at 35°C for 3 days
3. Observed a blue color which was changed from green due to alkalinization that means test is positive.

2.9.6: Methyl red test:

In methyl red test detects that microorganisms which can perform mixed acid fermentation after supply of glucose.

Media and Reagents:**Methyl red solution, 0.02%**

0.1 gm of methyl red which is already dissolved in three hundred (300 ml) of ethyl alcohol, 95 percent.

500 ml distilled water.

Stored at 4°C in a bottle.

Procedure:

1. before inoculation media was allowed to adjust at room temperature.
2. 24 hour cultured bacteria was taken and inoculate in medium.
3. Kept in incubator for 24 hours at 36 °C.
4. Added 3 drops of methyl red indicator to already incubated media.
5. Color changed to red was observed which mean test is positive

3. RESULTS

Culturing:

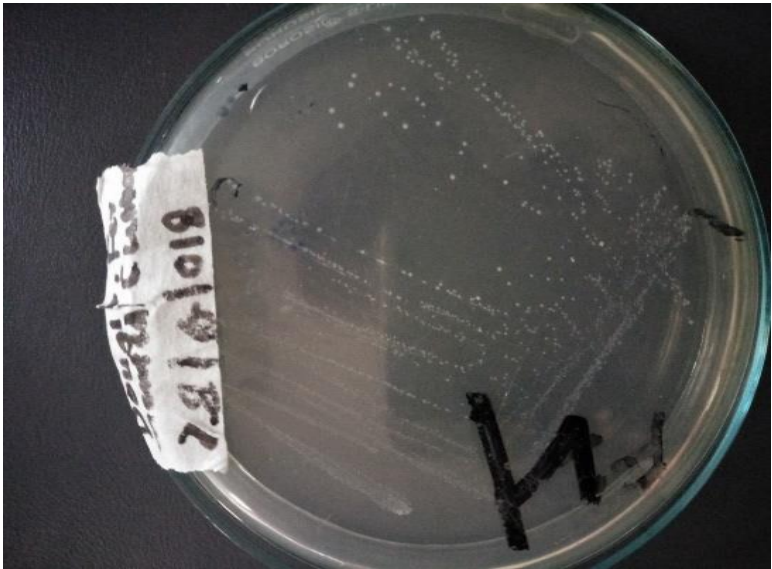


Figure 3-1: Culturing of dough by serial dilution using HS Media



Figure 3-2: Culturing of yogurt by serial dilution using HS media



Figure 3-3: HS culture media + fish culturing

Colonies from these cultured petri plates were taken and added to fermented flasks which has already fish pieces and HS media.

Fermentation:

Fish pieces along with HS culture media and dough, yogurt culture taken in 150 ml flasks and kept in incubator at 30°C.



Figure 3-4: Fermented flasks

3.1: Collagen Extraction Optimization:

For the extraction of fish collagen, preliminary studies were carried out for the identification of suitable extraction parameters. The samples were set at 37°C and the changing pH was carefully observed and noted down for four weeks. Table 3.

Table 3-1: Optimization of pH during four weeks' duration

Samples	Day 0	Week 1 pH	Week 2 pH	Week 3 pH	Week 4 pH
Distilled Water + Fish	8	8	8	9	9
Culture Media + Fish	6	6	5	6	6
Yogurt + Fish	5	5	4	5	5
Dough + Fish	4	4	5	5	4

Centrifugation:

Firstly, samples from all four flasks were centrifuge at 4500rpm for 10 minutes at 4°C and then take soup and again centrifuge in Microspin at 10000rpm for 3 minutes.



Figure 3-5: A. Ultra-Centrifuge, B. Micro spin Centrifuge

3.2: Gel Electrophoresis

The extracted collagen protein profile was observed with commercially available sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE revealed that the collagen protein of fish had doublet pattern for $\alpha 1$ and $\alpha 2$ chains at corresponding to 145kDa and 132kDa respectively. The density for $\alpha 1$ twice as compared to $\alpha 2$.

The fish collagen consists mostly of α -chain as well as little amount of inter and intra molecular cross-linked components of α -chains; b (dimer) and c (trimer). This type of dimer and trimer were seen in collagen of bigeye snapper (Kittiphattanabawon et al., 2005), back drum sea bream, sheephead sea bream (Ogawa et al., 2003), and ocellate puffer fish (Nagai et al., 2002). That's why

it's proved that lycine derived cross link was present in the soluble collagen fraction of scales (Kimura et al.,1991).

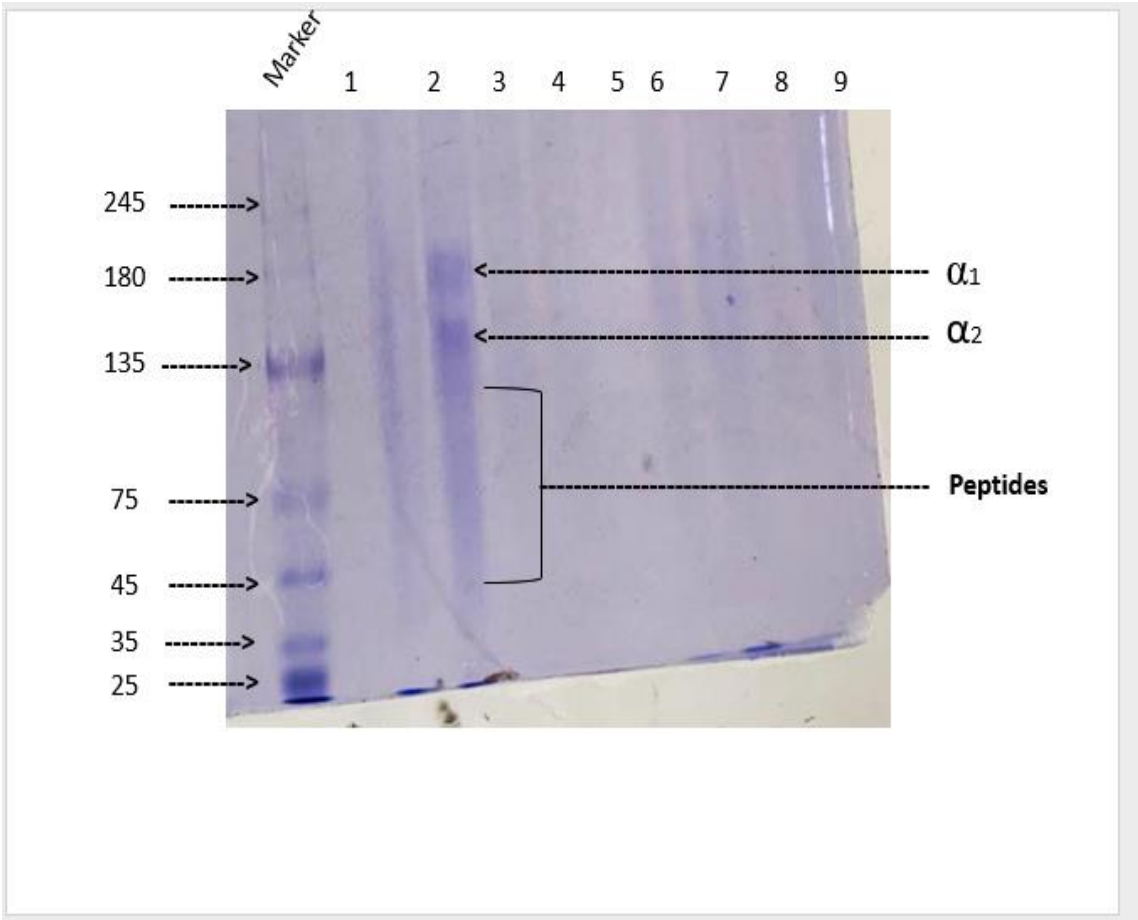


Figure 3-6: SDS-PAGE analysis of (1) distilled water +fish (2) yogurt +fish week1 (3) yogurt +fish week2 (4) yogurt +fish week3 (5) dough +fish week1 (6) dough +fish week3 (7) dough +fish week3(8) HS culture media +fish.

3.3 Biochemical tests:

Catalase test:



Figure 3-7: Catalase positive test

In catalase test four slides were taken and bacterial smear was formed. One drop of H_2O_2 was added. Bubbles were noticed for the results.

Urease test



Figure 3-8: Urease negative test

In urease test urease agar was used for this test. Slants were prepared and yogurt cultures were streaked on them. These slants were incubated for 24 hours. No Color change was observed after 24 hours.

Tsi (triple sugar iron agar) test:

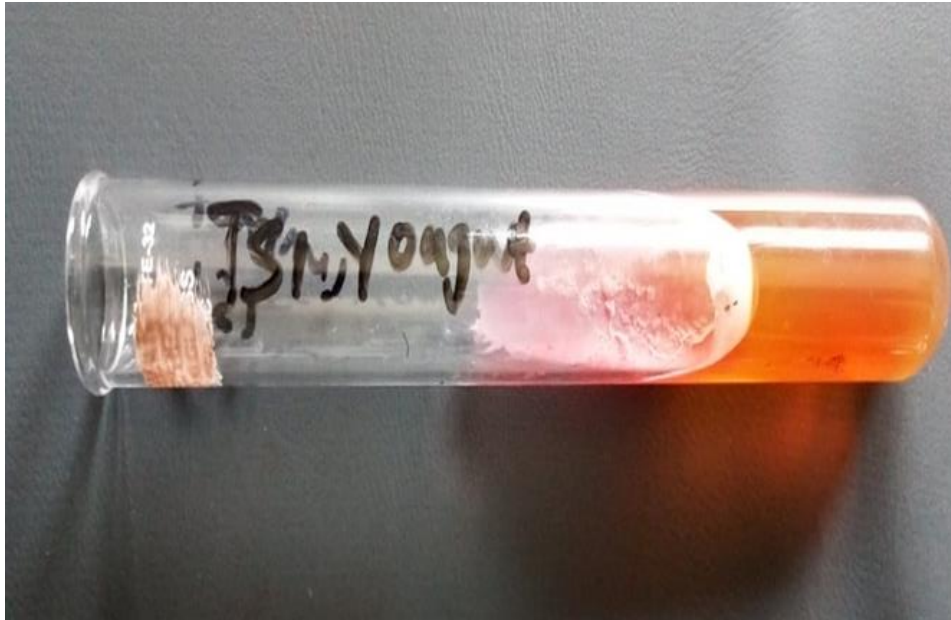


Figure 3-9: Tsi (triple sugar iron agar) positive

- In TSI test TSI slants were formed by using TSI agar and cultures were streaked on them. After 24 hours' incubation it was observed for color change.

Citrate test:



Figure 3-10: Citrate positive test

- In citrate test Simmons citrate agar was used to test either organism has ability to utilize citrate as the sole source of energy. This medium contains ammonium salts as nitrogen and citrate as carbon source. Slants were formed and cultures were streaked on them. Color change was observed after incubation for the results.

Indole test:

Figure 3-11: Indole negative test

In indole test peptone broth was prepared and autoclaved. Then cultures were inoculated into tube with sterile loop. These cultures were incubated for 24 hours. After incubation kovac's reagent (isoamyl alcohol, para Dimethylaminobenzaldehyd and concentrated HCl) was added in the culture broth. No red and violet rings were formed on the surface

which indicate positive so solution turns yellow. Variable, showing an orange color which is negative.

Methyl red test:



Figure 3-12: Methyl red positive test

- In methyl red test nutrient broth media was formed and poured in test tubes. Culture were added in these tubes and incubated for 24 hours. After incubation 5 drops of methyl red were added in the inoculums. Color change was observed for the results.

Cell Culturing:**Cell proliferation of HaCaT cell after treatment with fermented fish collagen peptides****Table 3-2: Treatment after 24hr**

24hr	control	Treated FFCP
	0.6	1.2
	0.65	0.9
	0.61	0.81
STD	0.264575131	0.204205779
SUM	0.531143783	0.778551445
%	100	146.5801672

Table 3-3: Treatment after 48hr

48hr	control	Treated FFCP
	1.2	2.1
	1.28	1.8
	1.23	2.3
STD	0.606217783	0.251661148
SUM	1.079054446	1.612915287
%	100	149.4748753

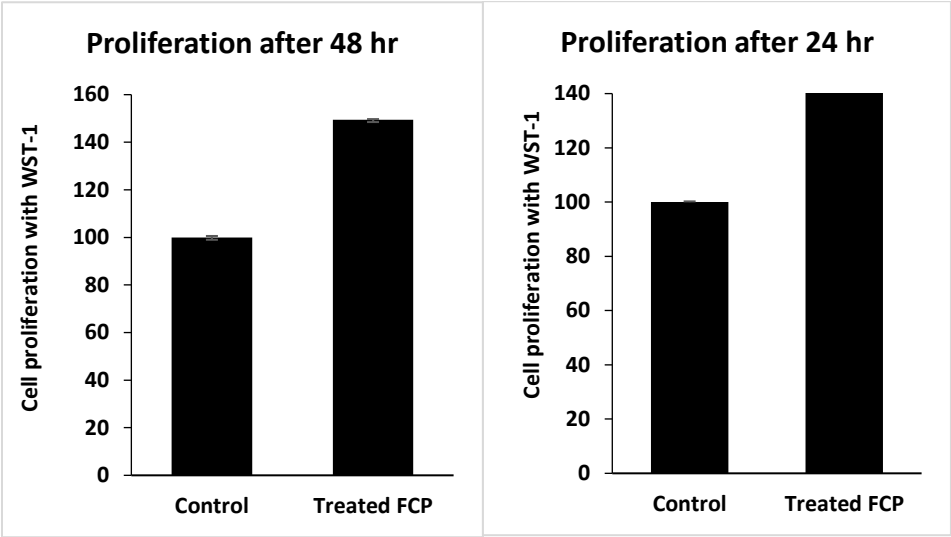


Figure 3-13: The human skin keratinocytes were culture in a 96 well plate with 1x10⁴ cell per well. The cells were treated with either HS culture media or with FFCP at the rate of 10 mg/ml for 24 and 48 hr respectively

4. DISCUSSION

Greek terms “kolla” means gum and “gen” means producing, have given the word collagen (Muralidharan *et al.*, 2013). Connective tissue has collagen as the most vital protein, and all multi-cellular species possess it in several forms (Schmidt *et al.*, 2016). Bone, skin, cartilage, smooth muscle and basal lamina has collagen, which is composed of a family of proteins. ECM and connective tissue possess collagen, the vital fibrous glycoprotein which hold the structural integrity of these tissues (Sandhu *et al.*, 2012). In human recently 28 different proteins have recognized as collagen (Vrana *et al.*, 2008). As collagen is a vital protein and has properties like physical and chemical properties, non-toxic and biocompatible, that's why used in cosmetic, pharmaceutical industry, biomedical materials and as food additives in food industry (Tylingo *et al.*, 2016). Old age is main reason for the deficiency of collagen in our body which lead to the looseness of skin and other organs (Sibilla *et al.*, 2015). To fulfill the deficiency of collagen in our body, currently collagen has been isolated from the marine source. It can be used for medicine purpose and 3D biomedical engineering too (Choi and Kim, 2015). Some religious issues, primarily in Islam and Judaism and the outbreak of bovine spongiform encephalopathy usually known as mad cow resulted the non-use of mammalian collagen. Fish collagen has been substituted of mammalian collagen as a source of collagen proteins (Mahboob, 2015). Skin, bones, fins and scales of fishes such jellyfishes, squids, sponges, octopuses, fish offal and cuttlefishes are used as source of collagen proteins (Tamilmozhi *et al.*, 2013). Successful isolation of collagen has several applications in cosmetics, food science and biomedicine (Berillis, 2015).

The current study was designed to extract collagen from fish through fish waste fermentation under various conditions. The fish waste treated with yogurt and dough culture was analysed by SDS-PAGE (sodium-dodecyl sulfate polyacrylamide gel electrophoresis) using 6% resolving and 5% stacking gel. The collagen optimization and its cytotoxic studies were performed using HaCaT cell-lines.

It is well known that different strains of enterobacteriaceae and lactic acid Bacteria mostly spoil different food types. 0,12–15. although mostly studies on just one or two type of foods. in this study we also discuss lactic acid bacteria are most dominant bacteria which can spoil food waste at temperature between 28°C and 35°C. LAB is well known bacteria which can play an essential role in food preservation and fermentation by lowering PH and reproduce bacteriocins which can stop the production of different pathogenic microorganisms.so in our study that's why we used

dough and yogurt bacteria which is safe to use and also anti pathogenic bacteria. So the presence of LAB indicates change in PH So with increase in lacto bacillus PH increase (Newton and Gill, 1978). In our study we observe PH on weekly basis so in first week have much change in PH but with time the PH become stable. This might recognize high oil content and low PH cause by fish waste in relative anaerobic condition.

According to our result lacto bacillus isolation from yogurt and dough was more as compared to cheese. In yogurt and dough have at least 10 type of lactic acid bacteria yogurt and cheese has much importance bio medically as it is safe to use and its non-pathogenic. In cheese have dominant species of lactobacillus that is get from raw dairy products because these bacteria can grow in harsh environment (Torres-Llanez *et al.*, 2006). But according to our results about 10 different LAB strains from dough and yogurt was isolated. Decrease in different things like loss of probiotic growth in yogurt like presence of hydrogen, low PH, increase in acidity etc.

So all of the above studies are same to our study. Results of our study showed that *Bacillus subtilis* was one of the common specie isolated from yogurt and dough culture fermentation, although many studies are not same to our study.

The SDS-PAGE revealed that the collagen protein of fish had doublet pattern for $\alpha 1$ and $\alpha 2$ chains at corresponding to 145 kDa and 132 kDa respectively. The density for $\alpha 1$ twice as compared to $\alpha 2$. Our result agrees that The fish collagen consists mostly of α -chain as well as little amount of inter and intra molecular cross-linked components of α -chains; b (dimmer) and c (trimer). This type of dimmer and trimer were seen in collagen of big eye snapper (Kittiphattanabawon *et al.*, 2005), back drum sea bream, sheep head sea bream (Pati *et al.*, 2010), and ocellate puffer fish (Nagai *et al.*, 2002). That's why it's proved that lysine derived cross link was present in the soluble collagen fraction of scales (Pati *et al.*, 2010).

In general, temperature, pH, extraction time and the acid influence affect the yield of collagen. Raise in temperature and increase in time improved the yield of collagen, until it reached to optimum level of 37° (Gudmundsson and Hafsteinsson, 1997). We optimized the production of fish waste fermentation using different temperature form 30°C to 37° C. On the other hand, declining of yield production starts after optimum conditions as the collagen is thermos-stable and denatures easily at room temperatures due to its chemical structure (Pang, 2016). The conformation of collagen may alter with high temperature for longer time that could reduce the solubility of collagen. Thus, collagen yield declines with rising temperature and lengthening the time. The

amino acids composition of the fish collagen varies between species. The collagen with a lesser hydroxyproline amount exhibits a lesser thermal stability as compared to its higher amount in collagen (Karim and Bhat, 2009a).

Moreover, the pH concentration also contributes the collagen's extractability. pH can control the protein's charge density that modify the structure and electrostatic interaction of the protein (Shen *et al.*, 2008). In current study, the pH for each sample was observed on weekly bases (see table 3). Our fermented fish collagen and peptides mixture were test for cellular cytotoxicity and proliferative effect on HaCaT cells. Our result shows that fermented extracted collagen are nontoxic and induce the proliferation of HaCaT cells. Our results are supported by various studies that revealed the medical application of fish extracted collagen as underline.

Similarly, fish derived collagen peptides were also determined to promote HUVEC proliferation and inhibited IL-6, IL-8, and TNF- α production in lipopolysaccharide-stimulated HUVECs (Zhang *et al.*, 2013). Recently, the marine collagen gained considerable attention because of their safety (Ólafsdóttir, 2003), and that become a key player in diverse biological properties, including antioxidant, anti-tumor (Ahmed *et al.*, 2015), anti-hypertensive (Zhang *et al.*, 2009), neuroprotective (Wong *et al.*, 2015), anti-skin aging and epiphyseal growth-promoting (Saito *et al.*, 2010), wound healing, and osteogenic and endothelial differentiation-promoting effects in rat bone marrow mesenchymal stem cells (Cavaliere *et al.*, 2015).

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