Alkali Hydrolyzed Conversion of Fish Scales into Protein Hydrolysates and Evaluation of their Antimicrobial Activity against Different Pathogenic Bacteria

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Summary: In this study the fish scales a waste product from the fish industry were converted into water soluble protein hydrolysates on heating with aqueous solution of sodium hydroxide. Those protein hydrolysates were fractionated into different fractions using solvent extraction procedure and were also checked for their antibacterial activity using agar well diffusion method. Their antibacterial activity was also tested against six pathogenic bacteria including *Escherichia coli, Margenella morganii*, *Haemophilus influenzae*, *Klebsilla pneumoniae*, *Pseudomonas aeruginosa*, and *Actenobactor baumannii*. All fractions were found active against these bacteria. Their zones of inhibitions were found to vary according to the nature of solvent fraction and bacteria. Maximum zones were observed for ethyl acetate fraction (neutral) against *Escherichia coli* and *Actenobactor baumannii* with zone of inhibition 19.5 mm and 17.0 mm, respectively almost equal to the control. Their chemical composition was also analyzed by using FTIR spectroscopy and spot test. These fractions and crude were found containing amides, amino acids, amines, phenols, alcohols and aldehydes functional groups. Their antibacterial activity was then correlated with their chemical composition along with their determination for percent yield and percentage of extraction.

Keywords: Protein hydrolysates, waste fish scales, antimicrobial activity, agar well diffusion method, FTIR spectroscopy

Introduction

The resistance of bacteria and fungi toward the antibiotics is rapidly increasing due to a number of reasons including excessive use of the antibiotics [1]. The chance of infection is far greater than earlier due to increasing population, rapidly change in life style and the presence of a number of wastes including food items and from food industry. According to an estimate annual municipal waste of Peninsular a Malaysian city is 8,196,000 tons, most of which is biomass from the food industry [2]. Fish farms and fish processing units is one of the food industries which is associated with a large number of waste. This waste is mainly composed of protein which is potential threat to the environment in terms of foul smell and as harbor for pathogenic bacteria and fungi. One of the important pollutant of fish industry are the fish scales which is more resistant to degradation due to its collagen nature [3]. It is due to their hardness, these are neither used by human being nor animals including dogs and cats [4]. These cause foul smell and also causing the aesthetic problems in addition to the dispersal of pathogenic microbes [5]. Their stiffness and hardness are responsible for slow degradation. Their incineration is associated with atmospheric pollution and is costly in terms of the wastage of useful carbon resource in addition to the incineration cost. An increasing trend of the integrated waste management and resource recovery has been observed to solve the waste management problems and recover and explore new resources [6].

One of the best options for the resource recovery from fish scales is its conversion into amino acids and peptides [3]. Where fish scales are mainly composed of type I collagen which can be hydrolyzed through the action of enzymes, acids or alkalis. Fish scales were converted into useful resource through solubilization of its collagen by the acids, buffers and enzymes [7]. Where collagen may be used in a number of applications including drug delivery, biomedical materials cosmetics and food [3,7-9]. Collagen and keratin wastes may be

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considered as potential source of protein and amino acids. A mixture of the collagen and keratin waste can be hydrolyzed into protein and amino acids using alkali hydrolysis [10,11]. Where alkali hydrolysis is considered as having no adverse effect on the collagen of the fish scales except its hydrolysis to some of the peptides [10,12]. The interactions of collagen with alkaline solution on heating involve the hydrolysis, degradation, rearrangement, re-polymerization and salt formation reactions. Some of the products may exist in the form of an equilibrium mixture. Solvent extraction may change the equilibrium concentrations which may sometimes results more changes in chemical composition [13]. Proteins, peptides and amino acids are reportedly antimicrobial in nature for example the use of collagen for food preservation is employed due to its antimicrobial properties [14-16]. The present work is intended to resource recovery, waste management and search of new antimicrobial peptides and amino acids combinations. The obtained products were analyzed for its functional groups using FTIR spectroscopy and chemical tests. The present work is mainly focused on the conversion of waste proteins into useful material. Another idea is the safe and economical disposal of this proteinaceous waste.

Experimental

Materials and Methods

Sample Solutions Preparation

The scales of *Cyprinous carpio* were obtained from a local fisherman of Mardan Khyber Pakhtunkhwa Pakistan. These scales were thoroughly washed with tap water to remove the stains of blood followed by washing with distilled water. After shade drying, scales were dissolved in 3% (0.75M) aqueous solution of sodium hydroxide with heating on a hot plate under reflux. It was then filtered after cooling and was used as a stock solution of fish scale samples.

Fractionation and Solvent Extraction of the Fish Scale Hydrolysate

The soluble protein hydrolysates solution that was obtained by heating of the waste fish scales with aqueous solution of sodium hydroxide was divided into three different fractions. Fraction 1 was named as alkaline fraction which was same as obtained from the dissolution of fish scales in 0.75 M sodium hydroxide solution. Fraction 2 was named as neutral fraction by neutralizing by addition of 2 M HCl in alkaline fraction. Fraction 3 was named as acidic fraction by acidifying the alkaline solution by the addition HCl until the pH reach to 2.54. Each of these three fractions was further fractioned by tituration with acetone and solvent extraction method using water immiscible solvents. In case of solvent extraction, 50 mL of each fraction was taken in the separatory funnel and extracted by 200 mL each of different solvents like *n*-hexane, chloroform and ethyl acetate in their ascending order of polarity *i.e.* first *n*-hexane, chloroform and ethyl acetate, respectively. Before tituration with acetone the aqueous solution of protein hydrolysate was concentrated by evaporation of water. This was followed by addition of acetone to dissolve the acetone soluble fraction. The resulting solution was filtered to separate the undissolved fraction. While the acetonic solution was allowed to vaporize to obtain the separated fraction. The case of acetone is little different from other solvents. It was employed for the removal of soluble contents from the hydrolysate concentrate which was obtained from the crude by evaporation of water using rotary evaporator.

Infrared Spectroscopic Analysis of the Fractions

The effect of changes on the chemical composition of the fractions of the fish scale hydrolysate was investigated using FTIR spectrophotometer by Shimadzu, Japan. The samples were analyzed by using ATR (attenuated total reflectance) of the spectrophotometer in transmittance mode. The scanning of the each extracted fraction as well as crude sample was carried out in the range of 400 to 4000 $cm.^{-1}$

Antimicrobial Assay of the Fish Scales Hydrolysate

The antimicrobial assays of the fish scale hydrolysate fractions and crude sample solutions were carried out against six human pathogenic bacteria and four fungi by agar well diffusion method [17]. These bacteria include *Escherichia coli*, *Margenella morganii*, *Haemophilus influenzae*, *Klebsilla pneumoniae*, *Pseudomonas aeruginosa*, and *Actenobactor baumannii*. All microbes were grown in a sterilized Luria bertini medium containing 12.5 g media, 10 g agar and 500 mL distill water in separate test tubes which were then placed in shaker incubator at 37 $^{\circ}C$ for 24 h to obtain approximately 10^8cfu/mL. Laria burtini agar medium was prepared for antibacterial. This media was autoclaved along with Petri plates, loop, cork borer, tips and glass rod at 121 $^{\circ}C$ for 20 minutes sterilization. The sterilized media was poured into petri dishes on cooling and shifted to the laminar flow hood under sterilized condition. The wells of 9 mm diameter were bored in each plate and

after solidification of the media the streaking of strains were carried out. The samples were applied in the form of a suspension of dimethyl sulphoxide (DMSO). It was prepared by dissolving 1 mg of the sample per ml of DMSO. It was applied on bacterial strains at the rate of 75 μL. Streptomycin and ampicillin were used as control during this study. Those plates were placed at 37 $^{\circ}$C for 24 h in incubator. The zones of inhibition were determined after 24 h. Each of the antibacterial assays was conducted in triplicate and the averages of results were reported.

Results and Discussions

Estimation of the Base Soluble Fraction of the Fish Scales

It can be seen from Table-1 that the total recovery of the hydrolysates fractions is greater in the basic medium *i.e.* 7.78% than the recovery of the hydrolysates fractions in neutral and acidic medium *i.e.* 6.78%, and 6.95%, respectively. It is because fish scales are mainly composed of type-I collagen. Type-I collagen can be hydrolyzed into soluble material using base hydrolysis [3]. This process is using sodium hydroxide as reactant and as facilitator of hydrolysis, terefore, some base is consumed during hydrolysis

Table-1: % Recovery of the fish scale hydrolysate fractions by solvent extraction and tituratration (Only acetone).

Solvent	Basic Fraction(g)	Neutral Fraction(g)	Acidic(g)
n-hexane	1.13	2.85	2.5
Chloroform	3.33	0.53	1.42
Ethyl acetate	1.82	2.49	1.79
Acetone	1.50	0.91	1.24
Total	7.78	6.78	6.95

It is used against the released of amino acids as a base and may result in the formation of salts. The consumption of base is partly due to the hydrolysis and partly due to the reaction of free amino acids and acid sites of peptides in salt formation [18]. It may also be used against the amino acids in reactions other than acid base reaction and most important of which is decarboxylation [19]. The evidence of this reaction can be seen in the FTIR spectra in Figure-1 which indicate the presence of amines, alcohols and phenols. It was observed that 8.25 % of the scales are dissolved per 100 ml of the aqueous sodium hydroxide solution. Although this dissolves fish scales into solution, however, the extraction efficiency is estimated in terms of the mass of the separated components which is obtained by the solvent extraction process. It can be also seen that the fraction extracted from the *n*-hexane is the least for basic media and greater for the neutral. Further variations can be observed and explained accordingly. This difference can also be seen in the FTIR spectra of these fractions.

FTIR Analysis of the Fish Scale Hydrolysates Fractions

FTIR spectra of the hydrolysate fractions obtained by solvent extraction for basic, acidic and neutral medium are shown in Figures 1-12. Figures 1-4 are the spectra for fractions obtained from basic solutions, scale and Figures 5-8 are for the neutral solutions of fish scales and Figures 9-12 for the acidic solutions. Each of this spectra have amides and amines as the common functional groups. However some of them contain other groups in addition to these indicating diverse chemical compositions. For example in case of basic solution *n*-hexane fraction shown in Figure-1, contains peaks for the amide, alkyne and hydroxyl group, that for chloroform fraction the spectrum shown in Figure-2, contain peaks for amines, amides, alcohol and phenols. In case of ethyl acetate the spectrum shown in Figure-3, indicates presence of amines, amides, alkanes, ketones, alcohol. Acetone fraction of basic hydrolysate the spectrum shown in Figure-3, contain Peaks for alcohol, nitro, amides and aliphatic amines.

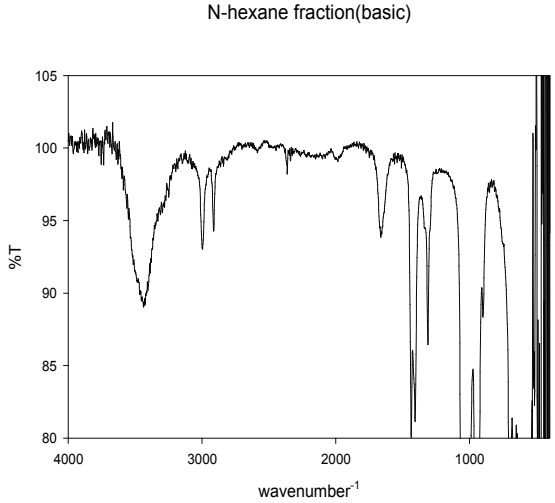

Fig. 1: *n*-Hexane hydrolysate fraction from basic medium.

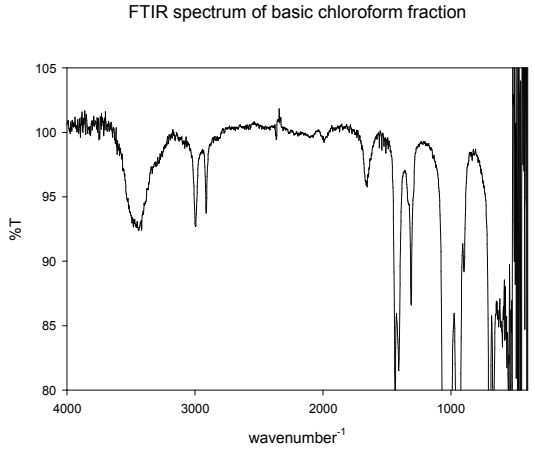

Fig. 2: Chloroform hydrolysate fraction from basic medium.

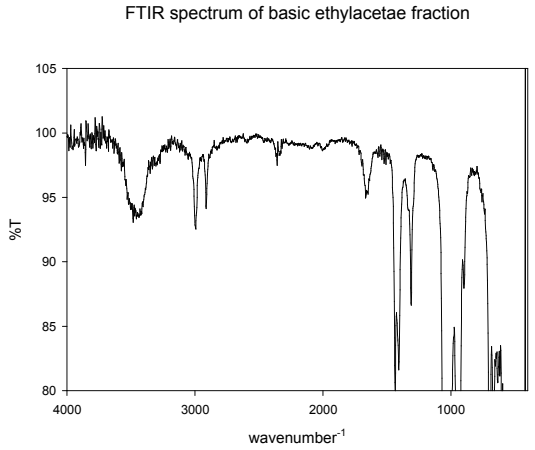

Fig. 3:: Ethyl acetate hydrolysate fraction from basic medium.

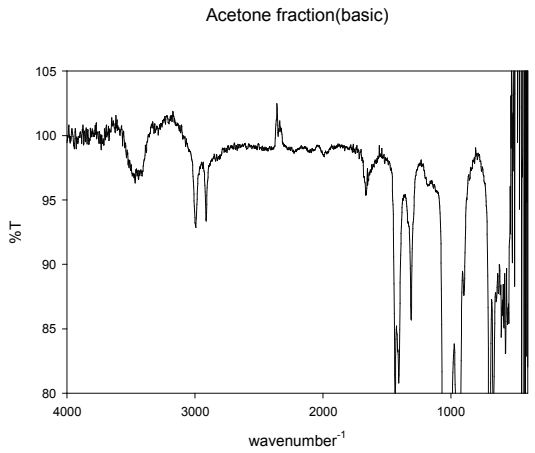

Fig. 4: Acetone hydrolysate fraction from basic medium.

Figure-5 is the FTIR spectrum of *n*-hexane fraction of neutral extract. It can be seen from the figure that this fraction contain alcohol, nitro group, amides, amines, and that for chloroform is given in Figure-6. This fraction is composed of amide, alcohol, phenols and amines. For neutral ethyl acetate fraction, the spectrum shown in Figure-7, was found to contain amides, ketones, phenols, alcohols and aromatic amines. The fraction obtained by tituration using acetone as solvent contain alcohol, amides, alkynes, nitro group and amines. Its FTIR spectra can be seen in Figure-8. The spectrum shown in Figure-8, was found to contain alcohol, amides, alkynes, nitro group and amines.

In case of the acidic hydrolysate the *n*-hexane fraction the spectrum shown in Figure-9 was found to contain amides, alcohols and phenols, chloroform fraction the spectrum shown in Figure-10, was found to have the same components as the *n*-hexane fraction. Ethyl acetate fraction the spectrum shown in Figure-11, was found to contain alcohols, phenols, amides and ketones in addition to aromatic amines. The spectrum shown in Figure-12 is for the fraction obtained by tituration with acetone. It can be seen from the spectra that this fraction contain alcohol, alkynes, amides and amines. The spectrum shown in Figure-12, was found to be composed of alcohol, alkynes, amides and amines.

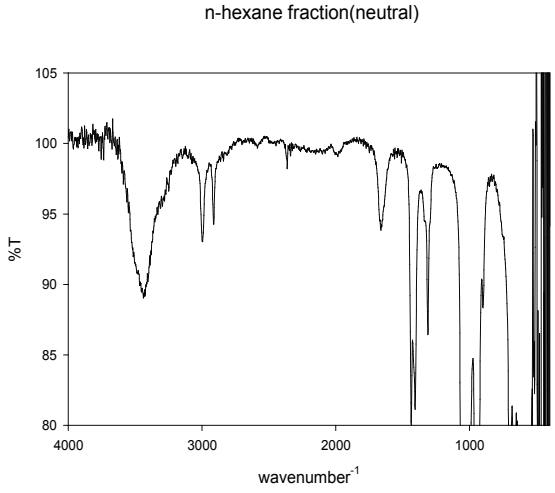

Fig. 5: *n*-Hexane fraction from neutral medium.

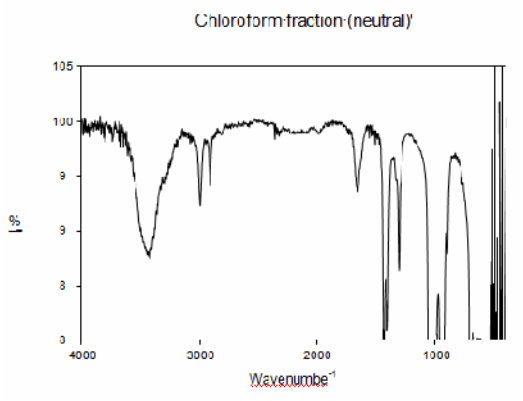

Fig. 5: *n*-Hexane hydrolysate fraction from neutral medium.

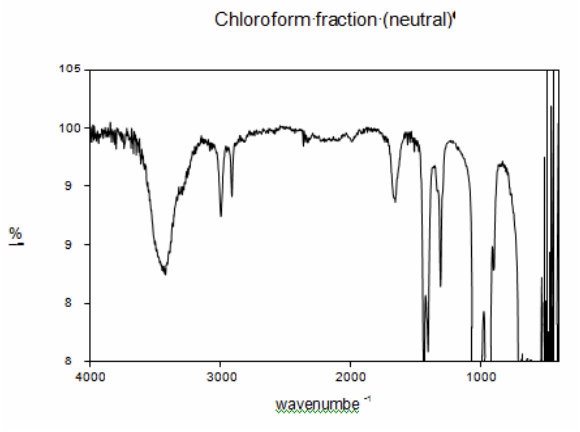

Fig. 6: Chloroform fraction from neutral medium.

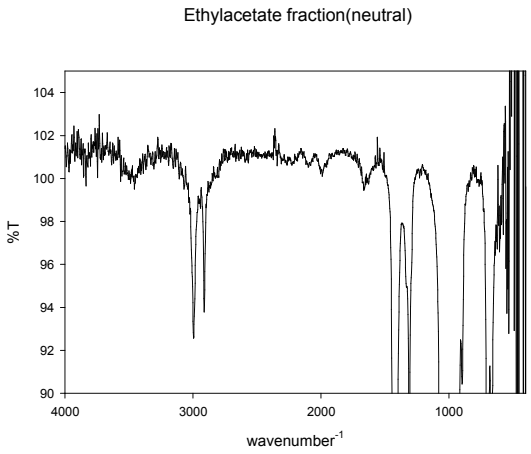

Fig. 7: Ethyl acetate fraction from neutral medium.

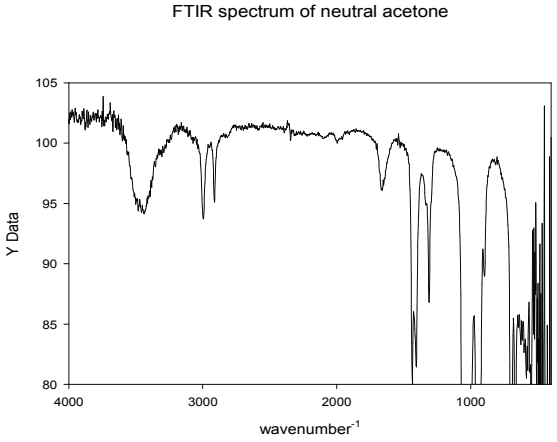

Fig. 8: Acetone hydrolysate fraction from neutral medium.

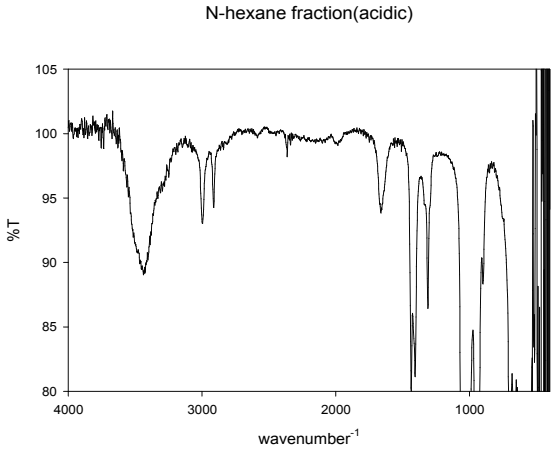

Fig. 9: *n*-Hexane hydrolysate fraction from acidic medium.

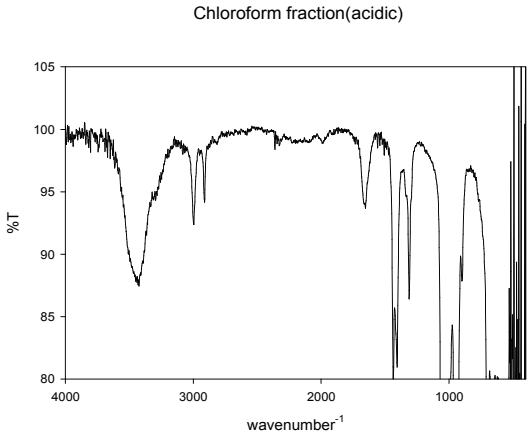

Fig. 10: Chloroform hydrolysate fraction from acidic medium.

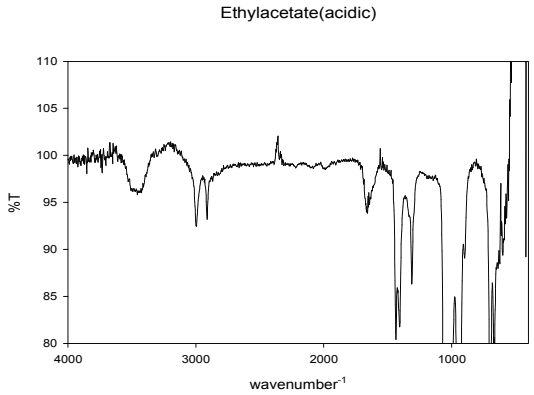

Fig. 11: Ethyl acetate hydrolysate fraction from acidic medium.

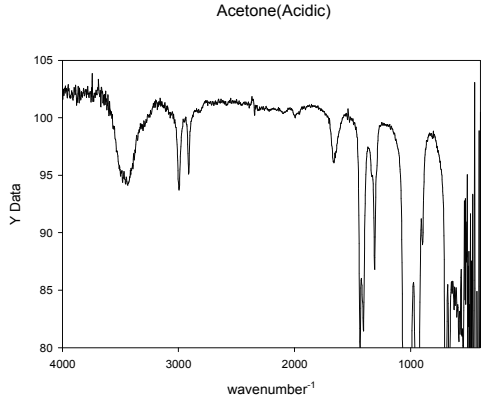

Fig. 12: Acetone hydrolysate fraction from acidic medium.

Comparison of the Chemical Composition of Extracted Fractions of Hydrolysates

The fractions obtained by the solvent extraction of each of the basic, neutral and acidic hydrolysate were compared for their chemical composition. The presence of amides is common in the *n*-hexane fraction of basic, acidic and neutral hydrolysate. However, the relative concentration varies according to the media. It is greater for acidic media having IR intensity of 59.08% the concentration of amides in basic media are slightly different with IR intensities of 71.6% and 74.17%, respectively. It is also expected that the hydroxyl group of *n*-hexane fraction is due to the unsaturated alcohols, however, the neutral and acidic is saturated alcohol. The concentration of these may also vary as can be seen from the intensity 61.5% and 58.2%, respectively. The basic *n*-hexane fraction does not contain phenol while acidic and neutral have significant concentration of phenols. IR intensity for acidic and neutral *n*-hexane fractions is 62.7% and 60.7%, respectively. Chloroform extract also contain amides, common for all basic, neutral and acidic hydrolysate. Their concentration is greater in basic media with 70.47% intensity. In case of acidic and neutral media, it was 73.97% and 74.17%, respectively. Unlike the *n*-hexane fraction phenols are present in the basic and not found in the neutral fraction of chloroform. In case of the basic media its relative concentration is greater. The IR intensity for which is 56.85% while for the acid it is 60.53%. Further acidic fraction contains alcohols unlike the basic and neutral.

Amides and ketones are common in the ethyl acetate extract of the three fractions. However, the relative IR intensities vary as 76.76% and 73.94%. Phenols are found in the neutral and acidic fractions only. The relative concentration of phenol is greater in neutral media with 77.4% intensity as compared to 69.28% for the phenol contents of acidic media.

Acetone fraction of the basic, neutral and acidic media contains amides and alcohol. The relative peak intensities of these is 54.74 and 83.84%, respectively. In addition to these compounds acidic and basic acetone fraction contains almost the same quantity of amines with a maximum peak intensity of 69.39%. Amines are not observed in the neutral and basic fractions.

Chemical Analysis for Phenols in Extracts of Fish Scale Hydrolysate Using Spot Test

Investigation of the presence of phenols is necessary for explaining and understanding the antimicrobial properties of the fish hydrolysate and its fractions. All the fractions were tested for the presence of phenol group using Iron III chloride spot test. The results are presented in Table-4. It can be seen from the results that phenols were found in the crude and most of the fractions. The source of which is hydrolysis of the aromatic ring containing amino acids of the collagen. The results of spot test are negative for some of the fractions. This may either be due to the exhaustive extraction by a previous solvent of the series or due to the non-extractable and highly ionic form of the extract or non-polar nature of extracting solvent.

Table-2: Spot test results for detection of phenols in fish scale hydrolysate fractions.

S. No.	Sample	Result
1	Neutral fish scales crude solution	Positive
2	Ethyl acetate layer (neutral)	Positive
3	Acetonic titurated extract (neutral)	Positive
4	Chloroform layer (neutral)	Negative
5	Ethyl acetate layer (basic)	Negative
6	Ethyl acetate layer (acidic)	Positive
7	n-hexane layer (neutral)	Negative
8	Chloroform layer (basic)	Positive
9	n-hexane layer (basic)	Positive
10	n-hexane layer(acidic)	Positive
11	Chloroform layer(acidic)	Positive
12	Acetone Titurated Extract (acidic)	Positive
13	Acetone Titurated Extract (basic)	Positive

Antimicrobial Activities of the Fish Scale Hydrolysate

The antibacterial activities of acidified, basic and neutral hydrolysate fractions are given in Table-3.

Table-3: Antibacterial activities of the acidified, basic and neutral hydrolysates fractions.

Sample	*Escherichia coli*	*Margenella morganii*	Haemophilus influenzae	*Klebsilla pneumonia*	*Pseudomonas aeruginosa*	*Actenobactor*
Acetone (acidic)	17.5	15.5	15.0	-	-	16.5
Acetone (basic)	-	15.5	18.0	11.5	-	16.5
Acetone (neutral)	16.5	-	15.0	13.5	-	14.0
Ethyl acetate (acidic)	16.5	14.5	16.0	14.5	-	17.0
Ethyl acetate (basic)	11.5	15.5	17.0	-	12.5	12.5
Ethyl acetate (neutral)	19.5	13.5	14.5	11.5	11.5	17.0
n-hexane (acidic)	16.5	12.5	14.5	14.5	-	11.5
n-hexane (basic)	16.5	13.5	17.5	12.5	12.5	11.5
n-hexane (neutral)	12.5	14.5	13.5	11.5	-	14.0
Chloroform (acidic)	13.5	14.5	12.5	11.5	-	14.5
Chloroform (basic)	13.5	15.0	15.5	13.5	16.0	16.0
Chloroform (neutral)	-	11.5	16.0	13.5	-	14.0
Ampicillin	18.0	-	20.0	-	-	20.0
Streptomycin	15.0	18.0	17.0	16.0	-	15.0

It can be seen from the Table-3 that the acidified hydrolysate have significant antibacterial activity. Further the antibacterial activity of these fractions varies according to the nature of bacteria. For example acidic acetone titurated extract is most effective against *E. coli* with a zone of inhibition 17.5 mm as compared to 18.0 mm of ampicillin and 15.0 mm zone of inhibition for streptomycin. However no activity was observed for *Klebsilla pneumonia* and *Pseudomonas aeruginosa*. In case of *Actenobactor* the active fraction is the ethyl acetate activity which is almost similar to ampiciline and greater than streptomycin.

Unlike acidic acetone extract, basic extract is most effective against *H. influnezae* with 17.5 mm zone of inhibition, however no activity was observed in *P. aeruginosa* and *E. coli*. This was also found effective against *Acetonebactor* with a maximum zone of inhibition 16.5 mm. Basic acetone titurated extract was found to exhibit mild activity against *K. pneumoniae* with 11.5 mm zone of inhibition. This activity may be due to presences of alcohol, amides, alkanes and amine Neutral acetone extract was found effective against *E. coli* with 16.5 mm zone of inhibition and no activity was found for *P. aeruginosa*. However, the extract was mildly active against *Margenella morganii* and *K. pneumoniae* with zone of inhibition 11.5 mm, respectively. Neutral ethyl acetate extract is most effective against *E. coli*. However in *P. aeruginosa* very least activity was observed.

The activity of acidic *n*-hexane and ethyl acetate fraction is next to the acetone titurated extract and are almost similar to the reference. These may be due to the presences of amides, alcohol and phenols. These fractions are highly active against *E. coli* and *H. influnezea*. Mild activity was observed against *P. aeruginosa*. Among the extracts obtained under basic and neutral conditions the most effective was neutral extracts. Next in activity are the ethyl acetate and *n*-hexane fractions. Basic ethyl acetate fraction is most effective against *H. influenza* unlike its neutral fraction which is most active against *E. coli*. Basic *n*-hexane fraction was found effective against *H. influenza* while the neutral was found most effective against *Marginalia morganii*.

Correlation of the Antimicrobial Activities with Chemical Composition of the Fish Scale Hydrolysate Fractions

The crude and solvent extracted fractions were found antibacterial. The cause of this activity is the chemical composition and combination. These may be due to the presences of amides, alcohols and phenols and in some cases aldehydes and ketones in addition to the mentioned compounds. The relative composition of these varies according to the polarity of solvent and media. The activities of these classes of compounds are reported. For example some amides are inhibitors of Peptide deformylase and reportedly active against *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Escherichia coli* [19, 20]. The activity of this fraction may be partially attributed to the presence of amides. The peak for amide is expected to be due to some of the amino acids. The presence of phenol in the extract may also be contributed to this antibacterial activity. Phenols were reported to be active against some Gram negative, Gram positive bacteria and some fungi [21-24]. Alcohols are reported to have antimicrobial activity [25-27]. The antimicrobial activity of *n*-hexane fraction is due to the combine action and synergistic effects. Variations in relative concentrations lead to variation in the activity and even mode of action. Therefore, difference in activity is observed for the *n*-hexane fraction obtained at the basic and neutral conditions.

Economic and Environmental Impact of the Extraction of Antibacterial from Fish Scales Hydrolysate Fractions

Since fish scale is waste product of fish industry which is a potential threat to environment in a terms dispersal of microbes foul smell and degradation of aesthetic values. It has been converted into silage, however, the use of silage is limited which is liquid media though it is used for the production of biogas but easily contaminate and difficult in transportation [28]. It may also be used as source of collagen [29], however, its large scale use is not reported. Its degradation is difficult due to its stiffness. There are reports of enzymatic degradation of fish scales which is also associated with problems [6, 30]. In the present work it is hydrolyzed into water soluble product. This can be easily separated into value added products like antimicrobial agents. It's processing and separation is a low cost practice. It can be easily dissolved by the use of only 3% aqueous solution of sodium hydroxide which is a low cost chemical. Its separation is carried out using solvent extraction where the solvents can easily be recycled.

Conclusion

Alkali hydrolysis was found to be effective in conversion of waste fish scales into water soluble hydrolysates named as the fish scale hydrolysate. It's processing and fractionation is of low cost. The obtained solutions were successfully separated into various antibacterial fractions. The fractions were found to have antimicrobial activity due to the presence of compounds like phenols, amines, amides, ketones and alcohols as suggested by their IR spectra. The varying antimicrobial activity of different fractions was due to different concentration of these compounds.

References

1. Goossenserman, Matus Ferech, Robert Vander Stichele Monique. "Out Patitient Antibiotics Use in Europe and Association with Resistance: a Cross Nation Database Study" Lancet Group Esac. Project, **365**, (2005) 579-587.
2. Johari, Anwar, Saeed I.A, Haslenda H., Habib A., and Mat R., "Economic and environmental benefits of landfill gas from municipal solid waste in Malaysia." Renewable and Sustainable Energy Reviews 16, no. 5 (2012): 2907-2912.
3. Pati, Falguni, Basudam A., and Santanu D., "Isolation and characterization of fish scale collagen of higher thermal stability" Bioresour. Technol., 101, 10 (2010): 3737-3742.
4. Sharpe P.T., "Fish scale development, hair today, teethand scales yesterday". Current Biology. 11, 18 (2001): 751-752.
5. Sanil N.K., and Vijayan K.K., "Diseases in ornamental Fishes" Department of Fisheries Thiruvanantha puram, (2008): 175-189.
6. Angell, Linda C., and Robert D., Klassen. "Integrating environmental issues into the mainstream: an agenda for research in operations management." Journal of Operations Management 17, no. 5 (1999): 575-598.
7. Nomura, Yoshihiro, Hiromitu Sakai, Yasuhiro Ishii, and and Kunio Shirai. "Preparation and some properties of type I collagen from fish scales." *Bioscience, biotechnology, and biochemistry* 60, no. 12 (1996): 2092-2094
8. Baar, Sibylle, Ch Schoerner, M. Röllinghoff, M. Radespiel-Tröger, H. P. Hümmer, and R. T. Carbon. "Collagen patches impregnated with antimicrobial agents have high local antimicrobial efficacy and achieve effective tissue gluing." *Infection* 29, no. 1 (2001): 27-31.
9. Kilian, Olaf, Hamid Hossain, Ingo Flesch, Ursula Sommer, Heiko Nolting, Trinad Chakraborty, and Reinhard Schnettler. "Elution kinetics, antimicrobial efficacy, and degradation and microvasculature of a new gentamicin-loaded collagen fleece." *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 90, no. 1 (2009): 210-222.
10. Gousterova, A., D. Braikova, I. Goshev, P. Christov, K. Tishinov, E. Vasileva-Tonkova, T. Haertle, and P. Nedkov. "Degradation of keratin and collagen containing wastes by newly isolated thermo actinomycetes or by alkaline hydrolysis." *Letters in applied microbiology* 40, no. 5 (2005): 335-340.
11. Cunningham, Leon W., John D. Ford, and Jere P. Segrest. "The isolation of identical hydroxylysyl glycosides from hydrolysates of soluble collagen and from human urine." *Journal of Biological Chemistry* 242, no. 10 (1967): 2570-2571.
12. Hattori, Shunji, Eijiro Adachi, Tetsuya Ebihara, Tomoko Shirai, Iori Someki, and Shinkichi Irie. "Alkali-treated collagen retained the triple helical conformation and the ligand activity for the cell adhesion via α2β1 integrin." *Journal of biochemistry* 125, no. 4 (1999): 676-684.
13. Pinto P.I.S, Estexao M.D., Redruello B., Socorro S.M., and Canario A.V.M., "Immune histo-chemical detection of estrogen receptors in fish scales" General and comparative Endocrinology, 160, 1(2009), 19-29.

14. Longfa J., "Preparation of Fish scale gelatin by mild hydrolysis and their characterization" J. Polym. Environ., 21 (2013), 564-567.
15. Pereda, M., A. G. Ponce, N. E. Marcovich, R. A. Ruseckaite, and J. F. Martucci. "Chitosan-gelatin composites and bi-layer films with potential antimicrobial activity." *Food Hydrocolloids* 25, no. 5 (2011): 1372-1381.
16. Cagri, Arzu, Zeynep Ustunol, and Elliot T. Ryser. "Antimicrobial edible films and coatings." *Journal of Food Protection*® 67, no. 4 (2004): 833-848.
17. Perez C., Pauli M., Bazevque P., "An antibiotic assay by the agar well diffusion method" Acta Biologiae et Medicinal Experimentalis. 15, (1990): 113-115.
18. Wang Y., Zhang C.L., Zhang O., "Composite electrospun nanomembranes of fish scale collagen peptides/chito-oligosaccharides: antibacterial properties and potential for wound dressing" International Journal of Nano medicine, 6, (2011): 667-676.
19. Cid, Sara B., Jesús M.A., Biserka B., Wilhelm H. Holzapfel, and Carmen M.V.C. "Amino acid decarboxylation by Lactobacillus curvatus CTC273 affected by the pH and glucose availability" Food microbiology, 25, 2 (2008): 269-277.
20. Hanake E., "Surgical treatment of in growing toe nails" Cutis, 37, (1986): 251-256.
21. Hanake E., "Controversies in the Treatment of In grow Nails" Dermatology Research & Practice, 2012, (2012): 1-12.
22. Hackbarth, Corinne J., Dawn Z., Chen, Jason G., Lewis, Kirk C., James B., Mangold, Jeffrey A., Cramer, Peter S., Margolis, "N-alkyl urea hydroxamic acids as a new class of peptide deformylase inhibitors with antibacterial activity" Antimicrobial agents and chemotherapy 46, 9 (2002): 2752-2764.
23. Genin, Michael J., Debra A., Allwine, David J., Anderson, Michael R., Barbachyn, D., Edward E., Stuart A., Garmon, David R., Graber, "Substituent Effects on the Antibacterial Activity of Nitrogen-Carbon-Linked (Azolylphenyl) oxazolidinones with Expanded Activity Against the Fastidious Gram-Negative Organisms Hemophilous influenzae and Moraxella c atarrhalis" Journal of medicinal chemistry 43, 5 (2000): 953-970.
24. Oliveira, Ivo, Anabela S., Isabel C. F., Albino B., Letícia E., and José A.P., "Total phenols, antioxidant potential and antimicrobial activity of walnut (Juglans regia L.) green husks" Food and chemical toxicology 46, 7, (2008): 2326-2331.
25. Sousa, Anabela, Isabel C.F., Ricardo C., Paula B., Andrade, Patrícia V., Rosa S., Letícia E., Albino B., and José A.P., "Phenolics and antimicrobial activity of traditional stoned table olives 'alcaparra" Bioorganic & medicinal chemistry 14, 24 (2006): 8533-8538.
26. Puupponen P., Nohynek R.R., Meier C., Kähkönen M., Heinonen M., Hopia A., and Oksman K.M. "Antimicrobial properties of phenolic compounds from berries" Journal of applied microbiology 90, 4 (2001): 494-507.
27. Sengul, Memnune, Hilal Y., Neva G., Bulent C., Zeynep E., and Sezai E.. "Total phenolic content, antioxidant and antimicrobial activities of some medicinal plants" Pak J Pharm Sci., 22, 1 (2009): 102-106.
28. Hong, Kyung H., Jong L.P., Hwan S., Ji Ho Y., and Tae J.K., "Preparation of antimicrobial poly (vinyl alcohol) nanofibers containing silver nanoparticles" Journal of Polymer Science Part B: Polymer Physics 44, 17 (2006): 2468-2474.
29. Bassolé, Imaël H.N., Aline L.M., Balé B., Souleymane T., Chlodwig F., Johannes N., Roger C.N., and Mamoudou H.D., "Composition and antimicrobial activities of Lippia multiflora Moldenke, Mentha x piperita L. and Ocimum basilicum L. essential oils and their major monoterpene alcohols alone and in combination" Molecules 15, 11 (2010): 7825-7839.
30. Kafle, Gopi K., Sang H.K., and Kyung I.S., "Ensiling of fish industry waste for biogas production: a lab scale evaluation of biochemical methane potential (BMP) and kinetics" Bioresource technology, 127, (2013): 326-336.