Investigation of the Effect of Base Strength on the Antifungal Activity and Chemical Composition of the Fish Scales Hydrolyzates

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Summary: The effect of base strength on the antifungal activity of the fish scale hydrolyzate was investigated for six types of samples prepared from the scales of *Cyprinus carpio* using sodium hydroxide in the range of 1-11% strength in the aqueous solution. Each of the sample was analyzed for its acid-base content using titration against HCl in addition to the spot test analysis for phenolic compounds. Each of these samples was analyzed using FTIR spectroscopy. Variation in chemical composition and functional group were observed with variation in the base strength. The *in vitro* antifungal activity of the fish scale hydrolyzates was tested against four pathogenic fungi including *Acremonium, Pythium, Verticillium,* and *Alternaria.* The antifungal assay was carried out using agar well diffusion methods. The sterilization was carried out using streptomycin while ketoconazole was used as the standard antifungal agent. Minimum inhibitory concentration was determined for the most active hydrolyzate which was obtained by 9% base solution. The cause of this antifungal activity was also discussed in this communication.

Keywords: Fish scale hydrolyzates, Antifungal activity, Agar well diffusion method, Spot test, FTIR spectroscopy

Introduction

Scales are protective armour for the fish and mainly composed of type I collagen [1-3]. Fish scales are included among the waste of fish farms and fish industry. This waste is difficult to manage because neither is it the food for cats and fishes nor it easily decomposed in composting and dumping [4]. This behavior is mainly due to the hard nature of these scales. Fish scales as solid waste may cause aesthetic problems by flowing here and there due to lightness and is considered as carrier of the pathogens [5, 6]. Fish scales disposal through the integrated approach of waste management seems an attractive option, due to its proteinaceous nature. It has been converted into protein and peptides by enzymatic hydrolysis **[**7]. It may also give the formation of a large number of amino acids including methionine, cysteine and glutathione on hydrolysis [8]. Its boiling along with other waste of fish farming may result the formation of silage [9]. Hydrolytic degradation of fish scales results the formation of amino acids and peptides [10]. The products of hydrolysis of collagen and proteins may depends upon the nature of protein as well as the reaction conditions including temperature, media and relative concentration of the acid or base [10, 11]. The resulting products may have a number of applications; these may be used as food additive, food supplement and source of the amino acids and proteins [8].

The present work is aimed to develop an easy, useful and economical method for the disposal of waste of fish industry *i.e.* fish scales. It is also aimed to evaluate the antifungal activity of the resulting amino acids and peptides obtained from the hydrolysis of these scales. This study is also aimed to study the effect of reaction conditions on the nature of product and its antifungal activity. Active fractions and nature of the product were also investigated.

Experimental

Material and Method

Fish scales were collected from a fish farm/shop in the Mardan City of Khyber Pakhtunkhawa Pakistan through a random collection. Three types of the fish were sold and processed by that retailer; the Rohu, Grass carp and Catla. The scales were predominantly composed of Grass carp. The scales were properly cleaned from the blood using tap water and then distilled water and were dried in the hood at the ambient temperature. Clean and dry fish scales (50) (250 g) were dissolved by heating in enough quantity of aqueous solution of sodium hydroxide to obtain a final solution of one liter. The aqueous solutions were used in the

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concentration range of 1-9% with respect to sodium hydroxide. However, the concentration/weight of the fish scales was kept constant for each of the solution. Each of the solution was evaporated using water bath to obtain the hydrlyzate for further use.

Antifungal activity of fish scale

Material

Test sample, potato dextrose agar, fungal strains, cork borer, micropipette, Petri plates (14 cm), spirit lamp, glass rod, organic solvent (DMSO), streptomycin was used for sterilizations and ketocanazole was used as standard. Incubator and Laminar flow hood were used during activity test.

Fungal Strains

Antifungal activity was tested against four pathogenic fungi including *Acremonium*, *Pythium*, *Veticillium*, and *Alternaria* and acquired from other researchers or cultured in lab.

Preparation of Culture media

For agar well diffusion method the antifungal susceptibility was tested by dissolving 39 g of potato dextrose agar in 1000 mL of distilled water. A small amount of streptomycin was also added to prevent test culture from bacterial contamination. It was placed on a hot plate with a magnetic stirrer until media is completely dissolved. The media was covered with aluminum foil and is sterilized by autoclaving at 121 $^{\circ}$C along with Petri plates, loop, tips and cork borer for 20 minutes.

Antifungal Fungal Assay by Agar Well Diffusion Method

The antifungal activities of fish scale extracts were determined using the agar well diffusion method [12]. The basic form of fish scale extracts were weighed and prepared in dimethyl sulphoxide at a concentration ranges between 1-6 mg/mL. The crude and separated fractions were tested against these strains in triplicate. The potato dextrose agar media was poured into plates under sterilized condition inside Laminar flow hood. On solidification of media, it was swabbed with fungal strains. After streaking wells of 9 mm in diameter were made with sterile cork borer in the plates. A 100 μL of each of the sample and standard ketocanazole were pipette out in each well and plates were incubated at 28 $^{\circ}$C for 48 to 96 h. The antifungal potential was evaluated according to the zone of inhibition against various pathogens. The zone of inhibition of sample as well as of standard was measured in mm.

Results and Discussions

Theoretical Basis of the Work

The aim of this work was safe and economical disposal of the fish scales which is a hazardous waste in terms of aesthetic problems and propagation of pathogens [13]. This waste may be converted into useful antimicrobial agent due to two reasons; it is believed that fish scales may have antimicrobial compounds and acts as protection for the fish [14]. It can be converted to antimicrobial agents due to its collagen composition. These may form peptides and amino acids under different conditions [15]. Literature reports indicate that some of the peptides may act as antifungal agents. There are peptides and amino acid that acts as antibacterial and even antiviral agents. The antimicrobial activities of these may depend upon the chemical composition and structural features of the proteins and peptides [16]. In the present work the concentration of sodium hydroxide was used as parameter controlling the chemical composition and structural features of the products of hydrolysis of fish scale collagen and protein. Variation in the concentration of sodium hydroxide may result the formation of monomeric and polymeric amino acid units of different chemical and structural properties that affect the antifungal properties of those.

Estimation of the Base Soluble Fraction of the Fish Scales

In view of the proteinaceous nature of fish scales its hydrolytic degradation was selected for its dissolution. The extent of degradation and the nature of products depend upon the concentration of solution. In this study this change was estimated in terms of the amount of sodium hydroxide utilized in different dissolution processes.

Amount of NaOH Utilized in Dissolution of Fish Scale

Fish scales are mainly composed of type I collagen. The reaction of collagen with base results hydrolysis of the collagen. The consumption of base is partly due to the hydrolysis and partly because of the reaction of free amino acids and acid sites of peptides in salt formation. It is also utilized in facilitating some chemical reaction like deamidation, rearrangement, cyclization and imidation [17]. The

amount of consumed base may give the extent of reaction and the amount of acid moieties. The extent of hydrolysis may change with change in strength of the base. It can be seen from the results in Table-1 that increasing strength of the base increased the amount of consumed base. This is due to the higher the hydrolysis and greater quantity of the acids produced in this process. This indicates difference in chemical composition of the hydrolyzed product and expected change in biochemical activity. The effect of pH on the hydrolysis and deamidation was investigated. This hydrolysis may involve imide ring formation and cyclic intermediate formation. The path way may change with variation in pH and other conditions [17]. Changes may occur in the nature of product or the extent of hydrolysis with variation in base concentration [9]. Literature reports indicated that proteins may pass through a number of modifications on reaction under oxidizing environment [19, 20]. It may pass through the hydrolysis of peptide linkage, carboxylation, hydroxylation or protein crosslinking.

In all the experiments, fish scales solution was titrated against primary standard HCl for determination of the unconsumed base in order to know that how much sodium hydroxide has been utilized during hydrolysis.

Table-1: Amount of NaOH used for dissolution of fish scale.

Wt of NaOH/100 mL	Wt of NaOH Used	% of base neutralized
1	0.892	89.2
3	2.64	88.0
5	4.28	85.0
7	4.156	59.28
9	6.048	67.22

Fish scales were dissolved on the basis of their hydrolysis by the sodium hydroxide. The resulting material is named as the crude hydrolyzate. The extent of the hydrolysis may vary with variation in the concentration of sodium hydroxide. It may also change the extent of solubility. The mass of dissolved substance may vary due to two reasons; Extensive hydrolysis may add greater quantity of water to the hydrolyzed collagen and results the formation of larger quantity of the dissolved product. Another reason is the extensive salt formation in addition to hydrolysis. This results the replacement of hydrogen ion of the amino acid by sodium ion of the sodium hydroxide resulting greater quantity of the resulting hydrolyzed product. It can be seen from the results in Table-2 that the amount of dissolved product increases with the increase in concentration of the sodium hydroxide. Fish scale solutions were selected on the basis of the initial concentration of sodium hydroxide employed for dissolution of the same quantity of fish scales. This difference in concentration of the sodium hydroxide is expected to change the composition of hydrolyzed and dissolved collagen of the scales. It can be seen from the table that increased concentration of the base increased the amount of extracted fractions which confirm the idea of difference in composition with increase in concentration of the base.

Table-2: Investigation of the mass fraction obtained through hydrolysis of fish scale.

Sample identity (% Sodium Hydroxide)	Weight of Fish scales + Base	Weight of Crude form (grams)	Weight Gain
1	5+1	11.92	5.92
3	5+3	13.93	5.92
5	5+5	15.79	5.79
7	5+7	18.1	6.1
9	5+9	16.1	4.1

FTIR Analysis of the Crude Fish Scale Hydrolyzates

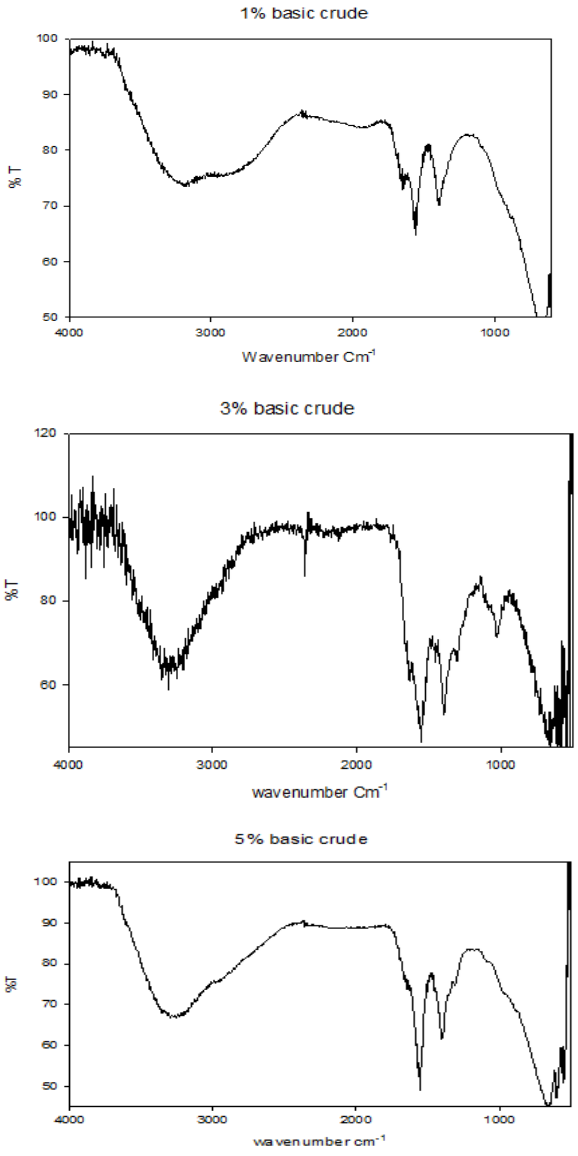
The chemical composition of the products of hydrolysis of proteins and peptides may vary according to the concentration of base. The interaction of collagen with base on heating involved the hydrolysis, degradation, rearrangement, re-polymerization and salt formation reactions. Some of the products may exist in the form of an equilibrium mixture. The effect of changes in chemical composition of the fractions of the fish scales was investigated using Fourier transform infrared spectroscopy. The analysis was carried out in transmittance mode. The scanning of each of the extracted fraction was carried out in the range of 500 to 4000 cm^{-1}. Prominent changes in composition were observed with change in concentration of the base strength.

FTIR spectra of crude hydrolyzate for basic medium are shown in Figs 1-5. Fig. 1 is the spectra of hydrolyzate obtained by 1 % sodium hydroxide. Absorption was observed at 1317.38, 1556.55, 1666.52, 2970.38, 3234.62 and 3350.35 cm^{-1}. These peaks may be attributed to the amino acids and amines. For example 1317.38 cm^{-1} is the stretching frequency of amino group. While 1666.52 cm^{-1} may be the stretching frequency of the carbonyl group while 3350.35 cm^{-1} is characteristic of NH_2. The difference in composition of the hydrolyzate obtained by hydrolysis of fish scales with 3% sodium hydroxide is apparent from the FTIR spectra in Fig. 2. Prominent absorption can be observed at 1261.45, 1305.81, 1537.27, 1631.78, 1643.35, 2862.5, 2950.0, 3183.8, 3358.07 and 3412.08 cm^{-1}. Unlike 1% crude these peaks are characteristic of aliphatic and aromatic amines. However, no peak was observed for the carbonyl group which indicates absence of amino

acids. In addition to difference in characteristic peaks, difference was also observed in absorption intensity of the peaks like 1317.38, 1556.55, 16666.52, 2970.30 and 3350.35 cm^{-1} of 1% are similar to frequencies of 3 % (1305.81, 1537.27, 1631.78, 1643.35, 2950.0 and 3358.07 cm^{-1}) but different in intensities which may be considered due to the difference in strength of the base. However, in some cases like 3234.62 of 1% and 1261.45 of 3% have the same intensity. The IR spectra of sample obtained through 5% sodium hydroxide solution was found to absorb infra-red radiations at 1240.23, 1348.24, 1402.25, 1556.55, 1693.50, 1781.35, 2781.34, 3147.47, 3224.98 and 3404.43 cm^{-1}. The 1240.23 cm^{-1} is illustrated for the presence of OH stretching, 1348.24 cm^{-1} peak is the stretching frequency of NH_2 group and 1556.55cm^{-1} for aliphatic and aromatics amines. 1693.50 cm^{-1} and 2781.35 cm^{-1} is for imines and aldehyde stretching, respectively. Like 1 and 3% of the samples the 5% have no carboxylic group which means having no amino acid. However, the peaks at 1240.23 of 5% is similar in frequency to 1261.45 of 1% but different in intensity and the peaks 1348.24, 1556.55, 1693.50cm^{-1}, 3147.47, 3224.98 and 3404.43 cm^{-1} are similar in frequencies to both 1% and 3% but different in intensities. There is no peak in 5% sample which have the same intensity to 1 and 3% samples. However, all the peaks in 5% of sample are slightly different in both frequencies and intensities from 1 and 3% of sample. In case of 7% sample prominent peaks are observed at 1566.20, 1693.50, 2704.20, 3354.21 and 3454.51 cm^{-1}. Both the 5 and 7% of the sample consist of almost the same group i.e. alcohol, phenols, imines, aldehydes and amino group. However, differences were observed in the peak intensity of 7% although the absorption at 1566.20, 1693.50, 2704.20, 3354.21 and 3454.51 cm^{-1} are similar to 1, 3 and 5%. While 3147.47 of 5% and 3354.21 cm^{-1} of 7% are only the two different frequency having similarity in peak intensity. The 9% sample gives peaks at 1269.16, 1317.38, 1556.55, 2717.70, 2900.94, 3132.40 and 3317.56 cm^{-1}. The peak at1269.16 cm^{-1} is for alcohol stretching, 1317.38 cm^{-1}, 1556.55 cm^{-1} is for aliphatic and amino group, 2559.54 for carboxylic acid and 2717.70 cm^{-1} is for aldehyde stretching. The peaks at 3317.56, 3132.38 cm^{-1} of 9% show similarity in their intensity to peak 1556.20, 3454.51 cm^{-1} of 7% but different in their frequencies. The peak 1317.38, 2900.94, 3132.40 and 3317.56 cm^{-1} of 9% are similar in frequency to 1, 3, 5, and 7% samples but different in their relative intensities. Further absorption was observed for almost all the samples and most prominently for samples obtained by 7% and 9% in the range of 2500-3500 cm^{-1} as a broader peak. This is due to the presence of salt of carboxylic acid.

Investigation of the Presence of Phenols in Fish Scale Extract by Using Spot Test

The different concentrations of crude solution were analyzed for phenol. Phenol was tested using spot test of iron (III) chloride dissolved in HCl solution. The fractions along with the test result for phenol are listed in table 4. All of which show positive result for phenol.



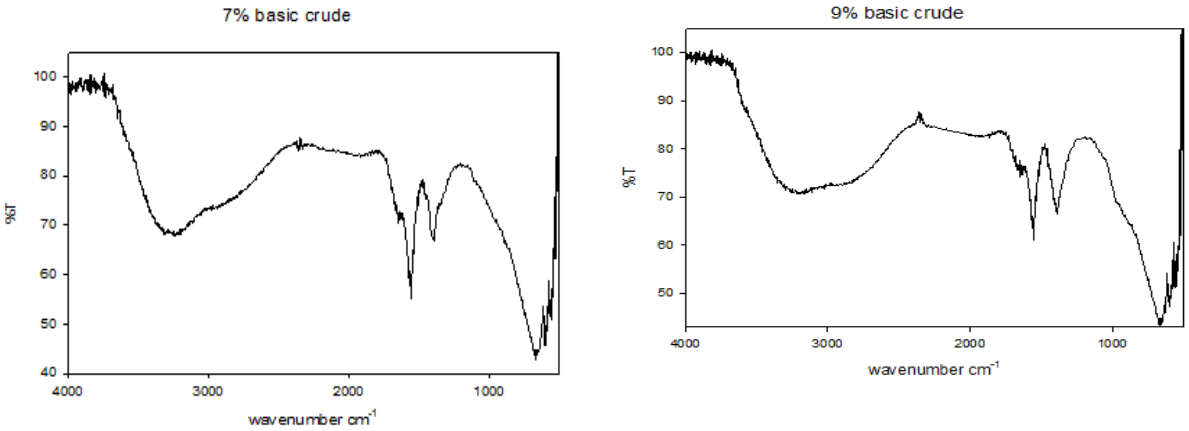


Fig. 1-5: FTIR spectra of crude hydrolyzate in basic medium.

Table-3: FTIR peak table for the protein hydrolyzates obtained at various base strengths.

% Con. of base	Wave number	Intensity	Functional group	Common	Additional compounds relative to 1%
1%	1317.38	78.18	Amines (C-N Stretching)	1	
	1556.55	64.68	Primary amines (N-H Bending)	2	
	1666.52	79.75	Carboxylic acid (O-H stretching)	3	
	2970.38	75.19	Alkane (C-H stretching)	4	
	3234.62	74.03	Alcohols/ phenols (O-H Stretching)	5	
	3350.35	76.57	Primary Amines (N-H Stretching)	6	
3%	1261.45	74	Alcohols (C-O Stretching)	5	
	1305.81	65.6	Amines (C-N Stretching)	1	
	1537.27	52.8	Aromatic (C=C stretching)	-	Aromatic (C=C stretching)
	1631.78	60.8	Primary Amines (N-H Bending)	2	
	1643.35	61.1	Tertiary amides (C=O Stretching)	-	Tertiary amides (C=O Stretching)
	3358.07	61.3	Alcohol/ phenols (O-H Stretching)	5	
	3412.08	65.8	Primary amines (N-H stretching)	6	
5%	1240.23	82.88	Alcohols (C-O Stretching)	5	
	1348.24	72.63	Amines (C-N Stretching)	1	
	1402.25	61.41	Alkane (CH3 Bending)	4	
	1539.20	59.36	Aromatic (C=C stretching)		Aromatic (C=C stretching)
	1556.55	48.8	Primary amines (N-H Bending)	2	
	1693.50	48.8	Imines (C=N Stretching)	-	Imines (C=N Stretching)
	2781.35	75.59	Aldehyde (C-H stretching)	-	Aldehyde (C-H stretching)
	3147.47	69.27	Aromatic (C-H stretching)	-	Aromatic (C-H stretching)
	3224.98	67.41	Alcohols/phenols (O-H Stretching)	5	
	3404.43	71.05	Primary amines (N-H stretching)	6	
7%	1292.31	79.14	Alcohols (C-O Stretching)	5	
	1313.52	76.61	Amines (C-N Stretching)	1	
	1566.20	57.8	Primary amines (N-H Bending)	2	
	1693.50	77.04	Imines (C=N Stretching)	-	Imines (C=N Stretching)
	2704.20	78.1	Aldehyde (C-H stretching)	-	Aldehyde (C-H stretching)
	3354.21	69.79	Alcohols/phenols (O-H Stretching)	5	
	3454.51	75.34	Primary amines (N-H stretching)	6	
9%	1269.16	80.96	Alcohols (C-O Stretching)	5	
	1317.38	77.39	Amines (C-N Stretching)	1	
	1556.55	63.48	Primary amines (N-H Bending)	2	
	2559.54	80.89	Carboxylic acid (O-H stretching)	-	Carboxylic acid (O-H stretching)
	2717.70	75.44	Aldehyde (C-H stretching)	-	Aldehyde (C-H stretching)
	2900.94	72.23	Alkane (C-H stretching)	4	
	3132.40	71.22	Primary amines (N-H stretching)	6	
	3317.56	72.2	Alcohols/phenols (O-H stretching)	5	

Table-4: Qualitative determination of phenols of the basic crude fractions.

% Conc. of base	Crude form Result
1	Positive
3	Positive
5	Positive
7	Positive
9	Positive

Investigation of the Antifungal Activity of Various Fractions Obtained From Fish Scale

Antifungal properties of the crude form of fish scale extracts using four strains of pathogenic fungi *i.e. Acremonium*, *Pythium*, *Verticillium*, and *Alternaria*. *Acremonium* is an opportunistic pathogen of human and animals, *Pythium* is pathogen of the animal while *Verticillium* and *Alternaria* are plant pathogens. The present report concerns itself with two variables: (i) change of antifungal activity of different groups with changes in chain length and unsaturation of compound due to change in concentration and (ii) the effect of these same compounds on different strains of fungi.

Fish scale hydrolyzates were found to have antimicrobial properties. These were found to vary with variation in the base strength used for hydrolysis and dissolution of the fish scales. This variation in the base strength was intended to change the chemical composition of the hydrolyzate. It can be seen from Table-5 that the hydrolyzate obtained by the use of 1% base is composed of primary amines, carboxylic acids, alcohol and phenol. Both the crude hydrolyzate and the extracted fraction were found antifungal. Further it was found that the activity of crude is greater than any of the extracted fractions. This can be explained on two theories. The crude is composed of the extra sodium hydroxide and some of its salt with organic moiety. Sodium hydroxide is a base and the antimicrobial properties of inorganic bases like calcium hydroxide are reported [21]. It has been mentioned that this crude hydolyzate contains salts of organic moieties. Where salts of the amines, phenol and carboxylic acids are reportedly antimicrobial [22, 23]. Antimicrobial properties of these crude hydrolyzates may also be explained on the basis of the symbiotic effect of the organic antifungal agents [24]. These agents include primary amines, phenol, alcohols and carboxylic acids and their salts [25, 26]. It can be seen from the table that the antifungal activity of the crude is almost near to that of the ketokenazole. This is mainly due to the symbiotic effect of the constituents of the hydrolyzate [25]. It was also due to difference in chemical composition that activity of the hydrolyzate obtained by the use of different base strength % was changed, as variation in base strength may change the nature of products of hydrolysis or the relative quantity of the products of hydrolysis of proteins and fats [27]. This variation in the relative quantity or the chemical composition may change the antimicrobial properties of the hydrolyzates. The IR studies of the protein hydrolyzate obtained by 3% base indicate that it is composed of tertiary amides in addition to primary amines, phenol, alcohols and carboxylic acids and their salts. It can be seen from table 3 that the hydrolyzate of 3% base was more active than 1% base. This is due to the extra amount of base which is 0.36 g and the presence of the tertiary amides where tertiary amides are reportedly antifungal [28]. The activity of the hydrolyzates obtained by 5% base is almost closer to the 3% base with slight variations. This might be due to presence of similar compounds. Slight variations in the activity may attribute to the presence of imines and aldehydes. These compounds are reported as antimicrobial [26]. The antifungal activity was found to increase with increase in the base strength used for hydrolysis. Significant changes in antifungal activities were observed with changes in the base strength at this high concentration of the base. This might be due to variation in the concentration of unused base and variation in the chemical composition. It can be seen from IR analysis that in comparison to hydrolyzate obtained by 1% base, it contains imines and aldehydes in addition to primary amines, carboxylic acids, alcohol and phenol. Further due to similarity in chemical composition with 3% base hydrolyzate, close similarity in antifungal activity was also observed. It can be seen from table 3 that the antifungal activity of 7 and 9 % base are closer. This is also due to the closer similarity in the chemical composition. Both of these extracts contain additional peaks for the carboxylic acids and aldehydes as compared to the 1% base hydrolyzates. The presence of carboxylic acid in relation to other agents acts as antifungal [29].

Table-5: Antifungal activity of the crude form of fish scale 6 mg/Ml.

Percent conc. of base	Zone of inhibition in (mm)			
	Acremonium	Pythium	Verticillium	Alternaria
0	0	0	0	0
1	16.0	11.0	13.0	11.2
3	20.5	20.0	14.0	15.5
5	22	22.6	15.5	16.0
7	22.5	21.0	17.0	16.0
9	24.5	23.5	16.0	18.0

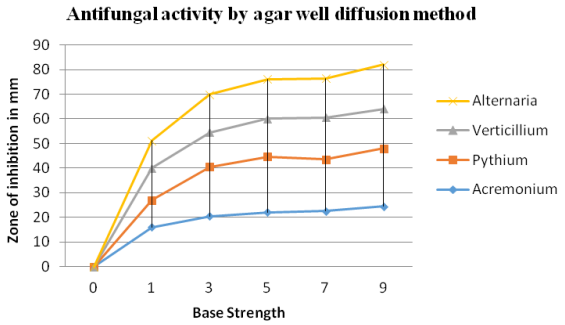


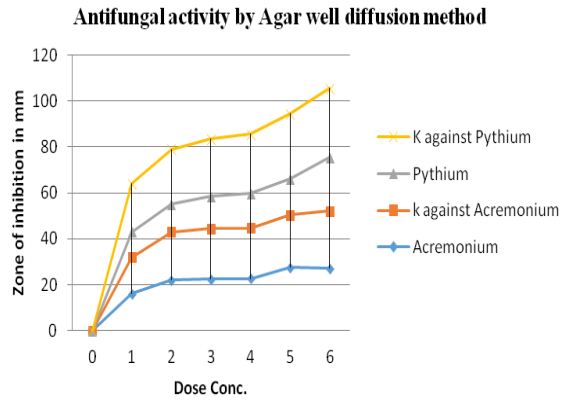
Fig. 1: Antifungal activity of fish scale hydrolyzate obtained at different base strength.

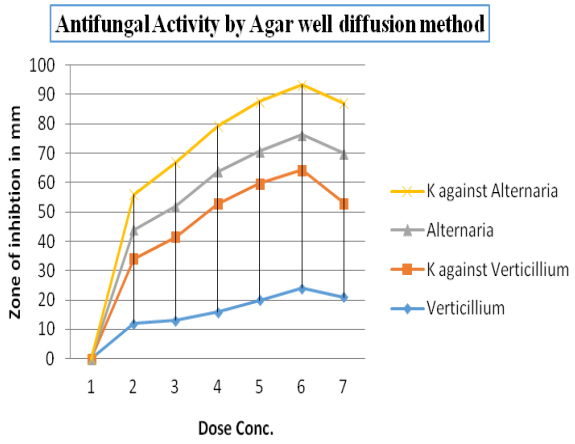
Investigation of the Proper Dose for the Antifungal Activity of the Crude Hydrolyzate of Fish Scale Protein

It was observed that the fish scale hydrolyzate obtained by hydrolysis with 9% sodium hydroxide give better results and is considered as the most active hydrolyzate. This hydrolyzate was selected for onward studies. The activity of this was given in table below.

Table-6: Antifungal activity of fish scale hydrolyzate obtained by 9% basic solution.

Wt of crude (mg)	K = Ketokenazole			
	Zone of inhibition = mm			
	Acremonium	K	Pythium	K
0	0	0	0	0
1	16.0	16.0	11.0	21.0
2	22.0	21.0	12.0	24.0
3	22.5	22.0	14.0	25.0
4	22.7	22.0	15.0	26.0
5	27.5	23.0	15.5	28.5
6	17.0	25.0	23.5	30.0





Antifungal activity of 9% basic crude at different Concentrations

Table-7: Antifungal activity of standard ketocanazole at different concentrations.

Conc. of crude (mg)	K = Ketokenazole			
	Zone of inhibition = mm			
	Verticillium	K	Alternaria	K
0	0	0	0	0
1	12.0	22.0	10.0	12.0
2	13.0	28.5	10.4	15.0
3	14.0	37.0	11.0	15.5
4	20.0	39.6	11.0	17.0
5	24.0	40.3	12.0	17.0
6	16.0	32.0	17.0	17.0

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