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Enzymatic degradation of white bovine pickled shavings yielding industrial gelatin and collagen hydrolysate

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Abstract

Six fungal species (T 1-6) and twelve isolates from bacterial fish wastes (BFW (1-7) and white pickled shavings wastes WPSW (1-5), excluded from Egyptian leather industry were examined for its utilization as a source for industrial gelatin and collagen hydrolysate. The conversion of the native/pretreated WPSW was conducted through protease blends in simultaneous processes. All the conducted studies confirmed the superiority and efficiency of the bacterial isolates in 2-d- shaken cultures at 37 ºC over all the tested fungal species under the proper conditions for proteases, especially collagenases productivity. The three bacterial isolates BFW5, WPSW3 and WPSW5 proved their prevalence as potent collagenase producers, which stimulates good gelatin formation through the controlled enzymatic hydrolysis of the two WPSW (native/pretreated) specimens, while at the same conditions, the isolates BFW7, WPSW3 & WPSW5 identified as (*Citrobacter freundii*) were nominated as potent hydrolysate producers. BFW5 identified as *(Bacilus cirus*) crude enzyme mixture surpassed all the others and afforded 42.5% lab scale gelatin formation efficiency, while the recorded hydrolysate % was only 5.97%. On the other hand, the bacterial isolate WPSW5 afforded the highest hydrolysate estimate (7.12%).

Keywords: Enzymatic degradation, white pickled bovine pickled, industrial gelatin, collagen hydrolysate

1. Introduction

Leather tanning industry releases a huge amounts of solid wastes, where one ton of raw material yields 250 kg of finished leather, the rest is mainly solid wastes as untanned raw trimmings (120 kg), tanned blue trimmings, pickled shavings, finished dyed and buffing dust (250 kg) [1-5]. The pickling step involves lowering the pH of the bovine skin in order to keep it for weeks or months before being tanned. The skin is pickled in a salt bath with sulfuric or formic acid. Obtaining a required skin thickness requires the pickled hide to undergo scraping resulting in huge amounts of white pickled shavings as a by-product. Based on the chemical point of view, the huge amounts of solid waste pieces issued from tanning, contain mainly collagen surrounded by non-proteinaceous materials, fats, salts and minor elements.

Utilization of these waste scraps, gives leather industry added values, and many trials have been done in this respect. The most usual use is to produce industrial and or nutritional gelatin. However, it is possible to maximize the benefits from these wastes by producing low molecular weight peptides (protein hydrolysate), which are widely used in cosmetics, health care and pharmaceutical applications [6-13]. Obtaining gelatin from untanned hides (pickled shavings) is a long-standing practice that, on a global scale, is carried out by means of inefficient chemical techniques, employing high water and energy consumption, yet they yield low collagen material, and often lead to a hard pollution impact and waste water load. Enzymatic methods have been also employed as very efficient and eco-friendly mean. However, combination of both chemical and enzymatic methods is also attained [14,15]. Chemical methods of collagen hydrolysis are carried out by means of strong acidic and alkaline conditions. Although, these methods are simple, they are cost-effective and environmentally un-acceptable [16,17]. Acidic treatment results in the structural unity unravelling and the cleavage of the noncovalent and intramolecular bonds. Materials

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with less intertwined collagen fibers such as fish and porcine skin are the preferred choice for the acidic process [18]. For the alkaline process, the raw materials are treated in basic solutions for a duration of a few days to weeks. The most commonly used process is the treatment of hard or thick substance that needs very aggressive penetration by aqueous sodium and calcium hydroxide solutions. Optimization of gelatin extraction was attained through response surface methodology [19].

Recently, enzymatic hydrolysis of collagen is used to optimize the yield of gelatin using mainly collagenase enzymes. The treatment time is much less than the required for alkali or acidic treatment and results in almost complete conversion to the pure product. The physical properties of the final gelatin are considered better [20]. The vast majority of researches have dealt with gelatin production by enzymes from animal bones Hosseini-Parvar*et et al.*, used an optimized conditions for enzymatic extraction of edible gelatin from the cattle bones using response surface methodology [18]. In 2019, Ma and his co-workers proposed a simple and eco-friendly method for gelatin production from bones as one-step biocatalysis [21]. A number of US patents have been published for the same scope [22-24]. Few researches have taken this from the leather shavings and fish wastes [25-30]. Other team used pepsin enzyme for pretreatment of bovine skin to improve gelatin recovery at the rate of 5, 15, and 25 units of enzyme/g of skin [31-33]; where the gelatin yield increased significantly. The present study focused on the optimized production of a microbial collagenase-rich protease blend effective on the pretreated pickled hide shavings yielding on a lab scale both industrial gelatin and collagen hydrolysate. The hydrolysate composition and structure will be discussed in a future manuscript.

Materials and methods

Leather waste (white pickled shavings, WPS)

WPS wastes were kindly provided from Elgabass tanneries in ELROBAKI, Cairo. In the present study the thick form was used for industrial gelatin production.

Microorganisms

Fungal species

Six fungal species were provided from the Culture Collection Centre of Chemistry (Natural & Microbial Products Dept., NRC, Cairo, Egypt), namely, *Aspergillus niger, A. terreus, Trichoderma viride, T. harzianum, T. reesie* and *Penicillium claviform.*

Bacterial isolates

A total of 12 bacterial isolates were isolated from fish and leather wastes according to the method published before [34].

Media

The following media were used in the present study and have the following composition (g/L):

Fungi maintenance and sub-culturing (medium 1)

Potato-dextrose agar (PDA) medium: potato infusion, 4.0 (infusion from 200 g potato slices); D-glucose, 20.0; agar, 15.0; pH 5.6 ±0.2. This medium was used for maintenance and sub-culturing of the fungal isolates and was supplied from Merck Corporation, Germany.

Fungal inocula preparation (Growth enhancement medium 2)

Glucose, 16; peptone, 5; yeast extract, 0.5; $MgSO_4.7H_2O$, 0.5 and KH_2PO_4, 1. This medium was used for fungal inocula preparation.

Bacterial maintenance and sub-culturing (Nutrient agar medium 3)

Meat peptone, 10; beef extract, 10; sodium chloride, 5; agar, 18; pH 7.3 ± 0.2. This medium was used for maintenance and sub-culturing of the bacterial isolates and was supplied from CONDA Company, Spain.

Bacterial inocula preparation (Growth enhancement medium 4)

Peptone, 5; NaCl, 5; yeast extract, 2 and beef extract, 1. This medium was used for bacterial growth enhancement.

Fungal collagenase enzyme production medium (medium 5)

$(NH_4)_2PO_4$, 1.0; $MgSO_4$. 7 H_2O, 2.0; KCl, 0.2, and the pH was adjusted to 7.5. Each 250 mL Erlenmeyer flask contains 50 mL medium was autoclaved, while the dehaired bovine-skin (WPS, pretreated/native) pieces (2 g of approximately 2×2 cm^2 size for each flask) were separately sterilized with ethanol, washed several times with sterile dist. water and added to the cooled autoclaved medium under aseptic conditions.

Bacterial collagenase enzyme production medium (medium 6), %, w/v

Glucose, 2.0; yeast extract, 0.15; tryptone, 1.0; $CaC1_2$, 0.005; $NaH_2PO_4.2H_2O$, 0.05 and KH_2PO_4. $3H_2O$, 0.25 and pH was adjusted to 7.5 [35]. The same steps were undergone as described above with bacterial collagenase production.

Pretreatment of WPSW

An amount of 300 g (WPSW) was immersed in 0.05 M NaOH (1:10, w/v) over night to enhance swelling and dissolve other non-proteinaceous materials, stirred for 48 h at 4°C, washed with water several times till neutrality then washed again with n-Butanol (500 mL) and EDTA (few grams) to remove fats and minerals, respectively [36].

Sterilization of WPSW

Two different methods were used for the sterilization process; the first was by autoclaving and the second was by soaking the desired weight of WPS (small pieces) in 70% ethyl alcohol for 3 h, under aseptic condition. Thereafter, the alcohol was decanted and the WPS washed with a sufficient amount of sterilized dist. water to remove the alcohol residues. Finally, the washed WPS was then added to the sterilized medium.

Isolation of bacterial isolates

Different bacterial isolates were isolated from some collected fish and leather waste samples according to Nagano and To [37], which were serially diluted (10^{-8}) by sterilized dist. water, and then 100 µL suspension were spread evenly on the plate, incubated at 37°C for 24 h. Collagenase activity was tested through a selective medium (pH 7.5). The selective medium contained (g/L), gelatin, 20; NaCl, 0.1; peptone, 5; KH_2PO_4, 0.5 and $MgSO_4$.$7H_2O$, 0.2: pH 7.5. After 24 h of incubation at 37°C, the acidic hydrargyrum solution was used to detect the colonies with gelatin hydrolysis activity. The acidic hydrargyrum solution consisted of 15g $HgCl_2$ and 20 mL dense hydrochloric acid per 100 mL. The bacteria with a bigger ratio of gel hydrolysis collar to colony diameter were inoculated into fluid medium. All the bacterial strains were streaked on to new medium plates before using acidic hydrargyrum solution for the detection of gelatinase activity. The selected positive isolates were grown in a liquid production medium [38].

Production of the fungal collagenases

All of the six fungal species were screened for collagenase enzyme production in the production medium 5, as previously described (in some cases the WPS waste and other medium constituents were autoclaved together), then inoculated with 48-h-old fungal inoculum. All the fungal species were incubated at 30°C for 5 and 7 days at agitation speed of 150 rpm. The observation was begun after 48 h to detect the WPSW hydrolysis.

Production of bacterial collagenases

The bacterial inocula of a bigger ratio of gelatin hydrolysis zone were applied in the production medium 6. The same steps, mentioned above with the fungal collagenases production, were carried out using the 24–h-old inocula of the positive isolates. All the isolates were incubated for different periods at 37°C with agitation speed of 150 rpm. The observation was begun after 24 h to detect the digestion of goat skin. A total of 12 isolates were tested for the collagenase production.

Assays for different protease activities

Collagenase

The reaction mixture contained 10 mg of insoluble collagen, 0.8 mL of 50 mM-Tris-HCl containing 4 mM $CaCl_2$, pH 7.5, and 0.2 mL of enzyme solution. The reaction mixture was incubated at 30°C for 30 min. with continuous shaking, and was stopped at various incubation times by the addition of 1.0 mL of 0.1 N acetic acid. The initial increasing rate of the free α-amino groups was measured according to Lowry *et al*. [39].

The graphical relationship construction between the pure gelatin solution concentration (w/v) and the refraction value (%)

Refraction value (%) for any gelatin solution was measured by the automatic refractometer. The relationship was constantly regular and typical, as shown in the Figure 1.

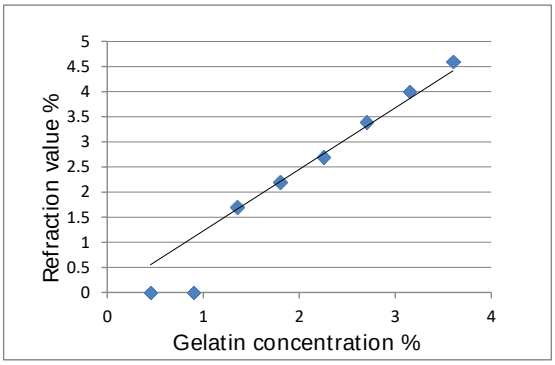

Figure 1: Refraction value (%) vs gelatin concentration (%)

Gelatinase

Gelatin was dissolved in boiled dist. water (0.2%, w/v). The reaction mixture contained 0.3 mL of the substrate solution, 0.2 mL of 150 mM Tris-HCI, pH 7.5, containing 12 mM $CaCl_2$ and 0.1 mL of enzyme solution. The reaction mixture was incubated at 30°C for 30 min and the reaction was stopped at various incubation times by addition of 0.6 mL of 0.1 N-HCl. The initial increasing rate of the free α-amino groups was measured as described by Awad *et al*. [40]. One gelatinase activity unit is defined as the amount of enzyme releases one µmol of tyrosine /1h reaction. Also, White and Fabian, 1953 method using Ostwald viscometer was applied to determine gelatinase activity as the % reduction

in 5% (w/v) gelatin viscosity due to the enzyme action.

Alkaline and neutral proteases

The enzyme activity was estimated in a reaction mixture containing 2.0 mL of casein solution (0.5%) in 0.1 M carbonate–bicarbonate buffer (pH 9.5 for alkaline and 7.0 for neutral protease, respectively), and 1.0 mL crude enzyme solution in a final volume of 3.0 mL. The reaction mixture was incubated at 40°C for 5 min., followed by termination of the reaction by the addition of 3.0 mL of ice-cold 10% (w/v) TCA (Trichloroacetic acid). After 1 h incubation at room temperature, the precipitate, so formed, was collected by the filtration of the contents using Whatman no. 1 filter paper. The tyrosine released by the action of crude enzyme on protein was estimated as described by Awad *et al*., 2011 [40]. One protease activity unit (Anson unit, A) is defined as the amount of enzyme that releases one µ gram of tyrosine per hour.

Protein measurement

Protein of any enzyme solution was measured applying Lowry *et al*., method [41].

Gelatin hydrolysate % determination

This was measured after enzymatic hydrolysis of the applied WPS in the clear supernatant of the reaction mixture according to Awad *et al*. [42].

Native/pretreated WPSW enzymatic hydrolysis time-course experiments

An amount of 2 g untreated or pretreated WPS waste were placed in 250 mL Erlenmeyer flask then add 5.0 mL of enzyme solution (culture filtrate) and the volume was completed to 50 mL with 0.2 M phosphate buffer, pH 7.0 followed by 2.5 mL of 0.1 M-NaN_3. Thereafter, then the flask was closed with fit stopper and incubated in a thermostatic shaker (180 rpm) at 37°C, after different time intervals (30, 48, 72, 120 h), the hydrolysis solution was taken to determine both of the produced gelatin and the hydrolysate.

Results and discussion

The first part concerns with exploring of the different biosynthesized proteases, namely collagenase, gelatinase, alkaline and neutral proteases by the chosen six fungi and twelve bacteria for the conversion of the native or pretreated WPSW to gelatin.

After different incubation periods, the biosynthesized crude enzymes by any organism are proteases mixture with variable efficiencies that leads to choose the most potent microbe producing the proper enzyme mixture. Under the adequate conditions the optimum production will be carried out by the potent microbe.

The known synergistic action of the produced proteases gathering to hydrolyze the WPSW protein components [41] justifies the determination of the individual productivity of each enzyme alone in the examined production cultures.

Table 1 displays the ability of the chosen six fungal species for collagenase enzymes production in shaken cultures at 30°C for 5 and 7 days incubation. All the tested fungi afforded either weak or no collagenase activity, however, both *Trichoderma ressei* and *Penicillium claviforme* afforded the highest productivity after 5 days (0.35 U/reaction) and the value will be later compared with the chosen bacteria productivity. Most of the tested fungal species produced collagenases at the early period (5 days) and the others at the late one (7 days).

Table 1 Survey of collagenase activity in some shaken fungal cultures utilizing native WPSW* after different incubation periods

Fungal species		Collagenase activity(U/reaction)**	
		Incubation period (day)	
		5	7
T. virdie	**(T 1)**	NA	0.15
T. harzianum	**(T 2)**	0.11	0.15
T. ressei	**(T 3)**	0.31	NA
A. niger	**(T 4)**	0.15	NA
A. Terreus	**(T 5)**	NA	0.11
P. claviforme	**(T6)**	0.31	0.05

*WPSW: white pickled shavings; **The enzyme reaction mixture composed of: 0.8 mL Tris buffer containing10 mg insoluble collagen + 0.2 mL enzyme solution (CF); NA: No activity.

Table 2 exhibited the alkaline proteases productivity by the six chosen fungi after 3 and 5-day-old shaken cultures. The two fungal species *T. reesei* and *A. terreus* produced highly active alkaline protease after 3 and 5 days incubation, while the other species exhibited either moderate (*A. niger* after 3 days) or no productivity (*T. viride*, *T. harzianum* and *P. claviforme*).

As reported in many articles, bacteria are consistently the most potent collagenase producers [42], the thinking tended to examine the ability of twelve bacterial isolates that were isolated from fish wastes (seven with symbol BFW1-7) and from WPSW (five with symbol WPSW1-5), the twelve unidentified bacterial isolates (BFW1-7 and WPSW1-5) were examined to explore their ability to produce collagenases and other proteases (Tables 2, 3, 5). It should be noted that the most potent

isolate/isolates will be completely identified at the end of the study.

Table 2 Survey of the alkaline protease production in some shaken fungal cultures utilizing native WPSW

Fungal species	**Alkaline protease activity (µg tyrosine/reaction)***	
	Incubation period (day)	
	3	**5**
T. virdie	NA	NA
T. harzianum	NA	NA
T. ressei	2633.04	2831.76
A. niger	412.34	NA
A. Terreus	8718.54	3974.4
P. claviforme	NA	NA

*The enzyme reaction mixture: 2.0 mL buffered casein solution (0.5%, w/v) + 1.0 mL enzyme solution (CF).

It was obvious that most of the bacterial isolates were able to enzymatically hydrolyze the leather waste after 2 days at 37 ℃ in shaken cultures and this was a prelude to evaluate their proteases productivity efficiency, which followed by the different protease activities measurement. It was noticed that the most potent are those isolated from WPSW and specifically the two isolates WPSW1 and WPSW5, which succeeded to hydrolyze most of the waste. The other fish-bacterial isolates performed strong or moderate leather hydrolysis, except for the isolate BFW4, which afforded weak action on the waste. These notices were taken into consideration in some experiments.

Table 3 displayed collagenase activity and gelatin refraction percent of 2 days shaken culture filtrates for the twelve bacterial isolates. The data revealed that all isolates except for BFW4 afforded high collagenase activities. Among all the tested isolates the isolate WPSW2 was the most potent and offered 18.23 U/reaction collagenase activity. It is worthy to note that in most cases the refraction percent measured by the automatic refractometer harmonized with collagenase activity.

It was crucial to explore the gelatinase activity in the filtrates of the fungal and bacterial cultures and therefore, gelatinase production in 5 days fungal cultures was recorded (Table 4). The fungal species *Trichoderma viride* offered strong gelatinase activity (0.73 U/reaction). The other five species showed either moderate or weak gelatinase productivity. It should be noted that strong gelatinase activity is unwanted as the gelatin is the main target of the present study.

Table 3 Survey of collagenase activity in some shaken bacterial cultures utilizing pretreated WPSW applying automatic refractometer after 2 days incubation.

Bacterial isolate	**Collagenase activity (U/reaction)**	**Gelatin formation (Refraction, %)**
BFW**1**	15.63	6.2
BFW**2**	NA	6.9
BFW**3**	15.37	5.3
BFW4	NA	5.0
BFW**5**	14.77	6.2
BFW**6**	16.52	6.1
BFW**7**	14.56	4.7
WPSW**1**	14.27	5.4
WPSW**2**	18.23	6.9
WPSW**3**	15.68	5.2
WPSW**4**	14.94	5.0
WPSW**5**	14.43	5.9

Table 4 Survey of gelatinase activity in some shaken fungal cultures utilizing pretreated WPSW and incubation periods 5 days

Fungal species	**Gelatinase activity (U/ reaction)***
T.virdie	0.731
T. harzianum	0.068
T. ressei	0.003
A.niger	0.338
A. oryza	0.494
P. claviforme	0.240

*The enzyme reaction mixture:0.3 mL substrate solution (0.2%, w/v) + 0.2 mL Tris buffer + 0.1 mL enzyme solution (CF).

The other enzyme activities afforded by the twelve tested bacterial isolates in 2 days shaken culture are recorded in Table 5. All bacterial isolates exhibited varied protease activities (gelatinase, alkaline and neutral proteases). The leather-bacterial isolates WPSW4 and WPSW5 and the fish-bacterial isolate

BFW3 exhibited the lowest gelatinase activities in their culture filtrates utilizing the WPSW. Among all the tested bacterial isolates, the isolate WPSW3 offered the highest levels of the three enzyme activities (0.94, 37290 & 17974 U/reaction, respectively). All other bacterial cultures possessed highly active alkaline and neutral proteases, which participate in the waste proteins hydrolysis.

Table 5 Survey of gelatinase, alkaline and neutral protease enzyme activities in some shaken bacterial cultures on pretreated WPS after 2 days incubation

Bacterial isolate	Enzyme activity (U/reaction)		
	Gelatinase	Alkaline protease	Neutral protease
BFW**1**	0.476	14779.80	4640.11
BFW**2**	0.818	15048.00	5921.86
BFW**3**	0.159	19578.89	5847.34
BFW4	0.846	15147.40	5931.8
BFW**5**	0.205	17462.50	5415.12
BFW**6**	0.912	14630.80	6190.13
BFW**7**	0.588	17159.50	5276.02
WPSW**1**	0.327	28163.60	15405.77
WPSW**2**	0.280	19439.80	11575.44
WPSW**3**	0.936	37289.80	17974.22
WPSW**4**	0.159	35337.40	15202.08
WPSW**5**	0.172	23588.00	11302.2

A group of experiments was conducted to study the hydrolysis time-course of the WPSW by the protease enzyme mixture of each bacterial isolate under specified conditions aforementioned in the Methods part. Accordingly, a group of mixtures containing a specific weight of dry native WPS waste with 5 mL crude enzyme (CF) of each isolate, 0.2 M phosphate buffer (pH 7.0) and 2.5 mL of 0.1 M NaN_3 in a total reaction mixture of 50 mL for different incubation periods (72 and 120 h) under shaking (180 rpm) at 37 ℃ and this was exploratory experiments applying the filtrates of the most potent 5 bacterial isolates (BFW5, BFW6, BFW7, WPSW3 & WPSW5). Both formed gelatin and hydrolysate soluble products were followed and determined.

The most promising bacterial shaken cultures were of 2-day-old and their filtrates were applied on the dry native WPSW soaked overnight on 0.2 M-phosphate buffer (pH 7.0) at room temperature. The enzymatic reaction mixture composed of 2 g soaked native WPSW, 5 mL crude enzyme mixture (CF), 45 mL 0.2M phosphate buffer and 0.25 mL 0.1 M NaN_3 in 250 mL Erlenmeyer conical flask well stoppered with rubber stopper and incubated in thermostatic shaker (180 rpm) at 37℃ for 3 and 5 days. Gelatin formed at period end was detected, well-dried and weighed in grams. Also, the soluble hydrolysate weight in milligrams was determined spectrophotometrically.

Table 6 displays both the formed gelatin and soluble hydrolysate products in each experiment after 3 and 5 days, respectively. It was declared that all the filtrates of the five potent bacterial isolates succeeded to affect the native leather wastes (WPSW) forming both gelatin and soluble hydrolysate products. Three days were quite enough for the bacterial isolates BFW6 and WPSW3 enzymes to form gelatin. Extension of hydrolysis period to 5 days led to complete hydrolysis of the formed gelatin. The data also revealed that the most efficient crude enzyme mixture was of the bacterial isolate BFW5 after 3 days that hydrolyzed the WPSW with 42.5% conversion efficiency. Moreover, it seemed that the formed gelatin began to hydrolyze as gelatinase (in the enzyme mixture) action on gelatin took place. This phenomenon was constantly recorded with all enzymes of five bacterial isolates. Furthermore, both BFW6 and WPSW3 filtrates hydrolyzed completely the entire formed gelatin after 5 days as mentioned before.

It is worth noting that, the hydrolysate product was measured only after WPSW hydrolysis operation for 3 days. Also, it was noticed that the overall relation between the formed gelatin and the hydrolysate product was reversed after 3 days WPSW enzymatic hydrolysis. This has been clearly confirmed by BFW5 crude enzymatic mixture, which surpassed all the others and afforded 42.5% gelatin formation efficiency, while the recorded hydrolysate % was 5.97%, which is lesser than those of BFW7, WPSW3 and WPSW5. The attached Figure below shows the ideal jelly formed by gelatin produced severally by enzymes of the potent isolates.

Table 6 Hydrolysis time-course of the native WPSW applying the most promising bacterial isolate filtrates forming experimental gelatin and hydrolysate products

Bacterial isolate (symbol)	Culture age (day)	Formed gelatin			Hydrolysate products
		Dry wt. (g/experiment*)	% (w/v) in 50 mL experiment	Efficiency (%) **	% (w/v) in 50 mL experiment
BFW**5**	3	0.85	1.70	42.5	5.97
	5	0.72	1.44	36.0	ND
BFW**6**	3	0.62	1.22	30.5	3.9
	5		Completely hydrolyzed		ND
BFW**7**	3	0.74	1.48	37.5	6.32
	5	0.47	0.94	23.5	ND
WPSW**3**	3	0.78	1.56	39.0	6.53
	5		Completely hydrolyzed		ND
WPSW**5**	3	0.75	1.50	37.5	7.12
	5	0.53	1.06	26.5	ND

*Total volume of experiment = 50 mL; **Dry native hydrolyzed WPSW = 2 g

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Conclusion

The conducted studies confirmed the superiority and efficiency of the bacterial isolates over all the tested fungal species under the proper conditions for proteases, especially collagenases productivity. The most important is the prevalence of the three bacterial isolates BFW5, *(Bacilus cirus*) , WPSW3 and WPSW5 in 2-d- shaken cultures at 37 ℃ as potent proteases, especially collagenase producers, which stimulates good gelatin formation, while at the same conditions, the isolates BFW7, WPSW3 & WPSW5 were nominated as potent hydrolysate producers. BFW5 crude enzyme mixture surpassed all the others and afforded 42.5% gelatin formation efficiency on lab scale, while the recorded hydrolysate % was only 5.97%. On the other hand, the bacterial isolate WPSW5 (*Citrobacter freundii*) afforded the highest hydrolysate estimate (7.12%). The produced lab scale gelatin is of industrial type.

REFERENCES

1. Mariod AA, Fadul H. Gelatin, source, extraction and industrial applications. *Acta Scientiarum Polonorum Technologia Alimentaria*, **12** (2),135-147 (2013).
2. Mohamed, O., Elsayed, H., Attia, R., Haroun, A., El-Sayed, N., Preparation of acrylic silicon dioxide nanoparticles as a binder for leather finishing, Advances in Polymer Technology, **37** (8), 3276–3286 (2018). https://doi.org/10.1002/adv.22112
3. Elsayed, H., Attia, R., Mohamed, O., Haroun, A., El-Sayed, N., Preparation of polyurethane silicon oxide nanomaterials as a binder in leather finishing, Fibers and Polymers, **19** (4), 832–842 (2018). https://doi.org/10.1007/s12221-018-7979-4
4. Elsayed, H., Hasanin, M., Rehan, M., Enhancement of multifunctional properties of leather surface decorated with silver nanoparticles (Ag NPs), Journal of Molecular Structure , **1234**, 130130 (2021). https://doi.org/10.1016/j.molstruc.2021.130130
5. Mohamed, O.A., Abd-El-All, A.S., Masoud, R.A., ElSayed, H., Activity of inorganic salts on different properties of synthetic leather, Egyptian Journal of Chemistry, **64** (7), 3971–3979 (2021). 10.21608/ejchem.2021.77438.3781
6. Elsayed, H., Ibrahim, S., Biodegradable package films of poly(L-Lactic) acid/extracted gelatin blend from white leather fibers, Egyptian Journal of Chemistry, **63** (8), 3059–3074 (2020). 10.21608/ejchem.2020.23131.2369
7. Ibrahim, S., Elsayed, H., Hasanin, M., Biodegradable, Antimicrobial and Antioxidant Biofilm for Active Packaging Based on Extracted Gelatin and Lignocelluloses Biowastes, Journal of Polymers and the Environment, **29** (2), 472–482 (2021). https://doi.org/10.1007/s10924-020-01893-7
8. Sultan, M., Elsayed, H., Abdelhakim, A.E.F., Taha, G., Active packaging gelatin films based on chitosan/Arabic gum/coconut oil Pickering nano emulsions, Journal of Applied Polymer Science, , 139 (1), 51442 (2022). https://doi.org/10.1002/app.51442
9. Zhang Y, Liu W, Li G, Shi B, Miao Y, Wu X. Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodonidella*). *Food Chemistry*, **103** (3), 906-912 (2007). https://doi.org/10.1016/j.foodchem.2006.09.053
10. Dang X, Yuan H, Shan Z. An eco-friendly material based on graft copolymer of gelatin extracted from leather solid waste for potential application in chemical sand-fixation. *Journal of Cleaner Production*, **188**, 416-424 (2018). https://doi.org/10.1016/j.jclepro.2018.04.007
11. Zhang Y, Tu D, Shen Q, Dai Z. Fish scale valorization by hydrothermal pretreatment

followed by enzymatic hydrolysis for gelatin hydrolysate production. *Molecules*, **24** (16), 2998-3012 (2019). https://doi.org/10.3390/molecules24162998
12. Zheng L, Wei H, Yu H, Xing Q, Zou Y, Zhou Y, Peng J. Fish skin gelatin hydrolysate production by ginger powder induces glutathione synthesis to prevent hydrogen peroxide induced intestinal oxidative stress via the Pept1-p62-Nrf2 cascade. *Journal of Agricultural and Food Chemistry*, **66** (44), 11601-11611 (2018). doi: 10.1021/acs.jafc.8b02840.
13. Al-Nimry S, Dayah AA, Hasan I, Daghmash R. Cosmetic, biomedical and pharmaceutical applications of fish gelatin/hydrolysates. *Marine Drugs*, **19** (3),145 (2021). https://doi.org/10.3390/md19030145
14. Skierka E, Sadowska M. The influence of different acids and pepsin on the extractability of collagen from the skin of Baltic cod (Gadusmorhua). *Food Chemistry*;**105** (3), 1302-1306 (2007). https://doi.org/10.1016/j.foodchem.2007.04.030
15. Kasankala LM, Xue Y, Weilong Y, Hong SD, He Q. Optimization of gelatine extraction from grass carp (Catenopharyngodonidella) fish skin by response surface methodology. *Bioresource technology*, **98** (17), 3338-3343 (2007). https://doi.org/10.1016/j.biortech.2006.03.019
16. Gao LL, Wang ZY, Zheng LI, Zhang CX, Zhang DQ. The characterization of acid and pepsin soluble collagen from ovine bones (Ujumuqin sheep). *Journal of Integrative Agriculture*, **17** (3), 704-711 (2018). https://doi.org/10.1016/S2095-3119(17)61751-9
17. Elsayed, H.M., Attia, R.Z., Mohamed, O.A., El-Sayed, N.H., Ibrahim, S.A., High bloom gelatin strength from white leather shavings, Leather and Footwear Journal, , **18** (4), 259–274 (2018). https://doi.org/10.24264/lfj.18.4.2
18. Hosseini-Parvar SH, Keramat J, Kadivar M, Khanipour E, Motamedzadegan A. Optimising conditions for enzymatic extraction of edible gelatin from the cattle bones using response surface methodology. *International Journal of Food Science & Technology*, **44** (3), 467-475 (2009). https://doi.org/10.1111/j.1365-2621.2008.01745.x
19. Norziah MH, Kee HY, Norita M. Response surface optimization of bromelain-assisted gelatin extraction from surimi processing wastes. *Food Bioscience*, **5**, 9-18 (2014). https://doi.org/10.1016/j.fbio.2013.10.001
20. Ahmad T, Ismail A, Ahmad SA, Khalil KA, Kumar Y, Adeyemi KD, Sazili AQ. Recent advances on the role of process variables affecting gelatin yield and characteristics with special reference to enzymatic extraction: A review. *Food Hydrocolloids*, 63, 85-96 (2017). https://doi.org/10.1016/j.foodhyd.2016.08.007
21. Ma Y, Zeng X, Ma X, Yang R, Zhao W. A simple and eco-friendly method of gelatin production from bone: One-step biocatalysis. *Journal of Cleaner Production*, **209**, 916-926 (2019). https://doi.org/10.1016/j.jclepro.2018.10.313
22. Rowlands AG, Burrows DJ, inventors; Eastman Kodak Co, assignee. Protease produced gelatin. United States patent US5919906A. 1999.
23. Knott Edward Bowes, photographic material, US4360590A, 1982.
24. Scott woodrow, Method of manufacturing gelatin of High purity required for imaging applications, US5908921A, 1999
25. Dalev PG, Simeonova LS. An enzyme biotechnology for the total utilization of leather wastes. *Biotechnol. Lett*; **14**, 531–534 (1992). https://doi.org/10.1007/BF01023181
26. Sundar VJ, Gnanamani A, Muralidharan C, Chandrababu NK, Mandal AB. Recovery and utilization of proteinous wastes of leather making: a review. *Reviews in Environmental Science and Bio/Technology*, **10** (2):151-163 (2011). https://doi.org/10.1007/s11157-010-9223-6
27. Ofori RA. Preparation of gelatin from fish skin by an enzyme (Doctoral dissertation, McGill University Montreal, Canada), 1999.
28. Kanagaraj J, Velappan KC, Babu NK, Sadulla S. Solid wastes generation in the leather industry and its utilization for cleaner environment-A review. *Journal of Scientific and Industrial Research*, **65**, 541-548 (2006). https://doi.org/10.1002/chin.200649273
29. Teodorescu M, Gaidau C. Possible steps to follow for filling the gap between requirements and the real condition in tanneries. *Journal of Cleaner Production*, **16** (5), 622-631 (2008). https://doi.org/10.1016/j.jclepro.2007.01.010
30. Hidayat G, Dewi EN, Rianingsih L. Characteristics of bone gelatin tilapia (Oreochromisniloticus) processedby using hydrolysis with phosphoric acid and papain enzyme. *Journal Pengolahanhasil Perikanan Indonesia*, **19**(1):69-78 (2016). https://doi.org/10.17844/jphpi.v19i1.11698
31. Ahmad T, Ismail A, Ahmad SA, Abdul Khalil K, Awad EA, Akhtar MT, Sazili AQ. Recovery of gelatin from bovine skin with the aid of pepsin and its effects on the characteristics of the extracted gelatin. *Polymers*, **13** (10), 1554 (2021). https://doi.org/10.3390/polym13101554
32. Ahmad T, Ismail A, Ahmad SA, Khalil KA, Kee LT, Awad EA, Sazili AQ. Physicochemical characteristics and molecular structures of gelatin extracted from bovine skin: effects of

actinidin and papain enzymes pretreatment. *International Journal of Food Properties*, **22** (1), 138-153 (2019). https://doi.org/10.1080/10942912.2019.1576731

33. Muhammad Y. S, Afifah A, Sharifudin M, Umi H. M. R, Characterisation of gelatin extracted from buffalo (Bubalusbubalis) bone using papain pre-treatment, *Journal of Agricultural and Food Engineering*, **4**, 0027 (2020). http://doi.org/10.37865/jafe.2020.0027
34. Wu Q, Li C, Li C, Chen H, Shuliang L. *Purification and characterization of a novel collagenase from Bacillus pumilus Col-J. Applied Biochemistry and Biotechnology*, **160** (1), 129-139 (2010). https://doi.org/10.1007/s12010-009-8673-1
35. Anal AK, Noomhorm A, Vongsawasdi P. Protein hydrolysates and bioactive peptides from seafood and crustacean waste: Their extraction, bioactive properties and industrial perspectives. Marine proteins and peptides: *Biological activities and applications*, **19** 709-735 (2013). https://doi.org/10.1002/9781118375082.ch36
36. Schmidt MM, Dornelles RC, Mello RO, Kubota EH, Mazutti MA, Kempka AP, Demiate IM. Collagen extraction process. *International Food Research Journal*, **23** (3), 913-922 (2016).
37. Nagano H, To KA. Purification of collagenase and specificity of its related enzyme from *Bacillus subtilis* FS-2. *Bioscience, Biotechnology, and Biochemistry*, **64** (1), 181-183 (2000). https://doi.org/10.1271/bbb.64.181
38. Liu D, Wei G, Li T, Hu J, Lu N, Regenstein JM, Zhou P. Effects of alkaline pretreatments and acid extraction conditions on the acid-soluble collagen from grass carp (Ctenopharyngodonidella) skin. *Food Chemistry*, **172**, 836-843 (2015). https://doi.org/10.1016/j.foodchem.2014.09.147
39. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.*, **193**, 265-275 (1951). https://doi.org/10.1016/S0021-9258(19)52451-6
40. Awad GE, Esawy MA, Salam WA, Salama BM, Abdelkader AF, El-diwany A. Keratinase production by Bacillus pumilus GHD in solid-state fermentation using sugar cane bagasse: optimisation of culture conditions using a Box-Behnken experimental design. *Annals of Microbiology*, **61** (3), 663-672 (2011). https://doi.org/10.1007/s13213-010-0187-0
41. Yan QJ, Huang LH, Sun Q, Jiang ZQ, Wu X. Isolation, identification and synthesis of four novel antioxidant peptides from rice residue protein hydrolyzed by multiple proteases. *Food Chemistry*, **179**, 290-295 (2015). https://doi.org/10.1016/j.foodchem.2015.01.137
42. Duarte AS, Correia A, Esteves AC. Bacterial collagenases–a review. *Critical Reviews in Microbiology*, **42** (1), 106-126 (2016). https://doi.org/10.3109/1040841X.2014.904270