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Mohammed & AL- Shamary

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PREPARATION OF PROTEIN CONCENTRATES FROM THE WASTE OF CARP FISH (*Cyprinus carpio)* AS AN ALTERNATIVE TO THE NITROGEN SOURCE FOR THE GROWTH OF LACTIC ACID BACTERIA

Ammar Shehab Mohammed[1*] ' Elham Ismael AL- Shamary[2]
[1]Department of Food Sciences, College of Agricultural Engineering Sciences, University of Baghdad, Baghdad, Iraq, Amar.Shehab1102a@coagri.uobaghad.edu.iq
[2]Assistant Professor PhD., Department of Food Sciences, College of Agricultural Engineering Sciences, University of Baghdad, Baghdad, Iraq, elham.ismael@coagri.uobaghdad.edu.iq

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ABSTRACT

This study aimed to prepare protein concentrates from carp fish (*Cyprinus carpio)* waste as an alternative to nitrogen sources for the growth of lactic acid bacteria. Two types of protein concentrates were prepared using the pH shift method (Acidic protein concentrate at pH 2/4.5 and Basic protein concentrate at pH 12/4.5). The results showed that the process of removing the fat and precipitating proteins at the isoelectric point contributed to increasing protein recovery in the protein concentrates, the number of *Lactobacillus acidophilus, Lactobacillus rhamnosus GG,* and *Bifidobacterium animalis* colonies in the alternative media was lower than the commercial DeMan, Rogosa and Sharpe (MRS) medium, the number of *Lactobacillus plantarum* colonies was approximated to the commercial media, while the number of *Bifidobacterium infantis* colonies was higher than the commercial media.

Keyword: Commercial media ,Culture media, Isoelectric point.

تحضير المركزات البروتينية من مخلفات اسماك الكارب *Cyprinus carpio* كبديل لمصدر النتروجين في نمو بكتريا حمض اللاكتيك

عمار شهاب محمد [1] ، الهام اسماعيل الشمري [2]
[1]قسم علوم الاغذية ، كلية علوم الهندسة الزراعة ، جامعة بغداد ، بغداد ، العراق ، Amar.Shehab1102a@coagri.uobaghad.edu.iq
[2]الاستاذ المساعد الدكتور ، قسم علوم الاغذية ، كلية علوم الهندسة الزراعة ، جامعة بغداد ، بغداد ، العراق ، elham.ismael@coagri.uobaghdad.edu.iq

الخلاصة

هدفت هذه الدراسة الى تحضير مركزات بروتينية من مخلفات اسماك الكارب *(Cyprinus carpio)* كبديل لمصادر النيتروجين في نمو بكتريا حمض اللاكتيك ، اذ تم تحضر نوعين من المركزات البروتينية باستعمال الطرائق القاعدية (المركز البروتيني الحامضي عند الرقم الهيدروجيني 4.5/2 والمركز البروتيني القاعدي عند الرقم الهيدروجيني 4.5/12) ، وأظهرت النتائج أن عمليتي إزالة الدهن وترسيب البروتينات عند نقطة التعادل الكهربائي اسهمت في زيادة استرجاع البروتين في المركزات البروتينية المحضرة ، وكان عدد مستعمرات *Lactobacillus acidophilus* و *Lactobacillus rhamnosus GG* و *Bifidobacterium animalis* في الاوساط البديلة أقل من وسط DeMan, Rogosa and Sharpe (MRS) التجاري، وعدد مستعمرات *Lactobacillus plantarum* قريبًا من الوسط التجاري، بينما كان عدد مستعمرات *Bifidobacterium infantis* أعلى من الوسط التجاري المستعمل.

الكلمات المفتاحية: الاوساط التجارية، الاوساط الزرعية، نقطة التعادل الكهربائي.

[*]**This article is taken from the doctoral dissertation of the first researcher.**

INTRODUCTION

Fish manufacturing processes produce many side streams with high nutritional value because they contain many nutritional elements such as proteins, fats, mineral and vitamins, but they cause potential environmental problems **(Aikaterini *et al.*, 2020)**, so many studies have aimed to benefit from them in preparing various products to reduce their environmental risks and , on the other hand, increase their economic value **(Salih *et al.*, 2021)**. Fish waste includes heads, frames, skin, trimmings, and viscera **(Khandakar, 2022)**. Carp (***Cyprinus carpio***) is a freshwater fish and is characterized by its ability to withstand different levels of temperature and dissolved oxygen, it is an omnivorous fish that feeds on various plant and animal sources, it grows quickly and reaches maturity after two years, therefore, it is of great economic importance in some countries, riverine in others **(Tessema *et al.*, 2020 ; Al-Hassani & Mustafa, 2022)**. Carp fish waste contains varying of protein as it constitutes (22.1, 13.9, and 25.9)% of scales, fins, and bones, respectively **(Maktoof *et al.*, 2020)**. Fish waste can be used to prepare protein concentrates using many methods that aim to exclude non-protein parts **(Sarojini *et al.*, 2021)**. The pH shift method is one of the most promising methods for producing protein concentrates as it is possible to obtain products with a high protein content and functional properties through it Better **(Abdollahi & Undeland, 2019)**. The pH shift method involves adjusting the pH to alkalini or acidic numbers to dissolve the highest amount of proteins, followed by precipitation of the dissolved proteins at their e isoelectric point **(Kakko *et al.*, 2022)**. **Jaber & Najim (2021)** prepared a protein concentrate from the viscera of common carp fish using acetic acid, and it contained (9.76, 71.7, 11.3, and 6.02)% moisture, protein, fat, and ash, respectively. **Meshre *et al.*, (2021)** found that protein concentrates prepared from pink perch (***Nemipterus japonicus***) by the pH shift method contained (54.57, 76.12, and 58)% protein at pHs (7, 11, and 3) respectively. It is also possible to improve the characteristics of protein concentrates prepared by the pH shift method by combining them with other methods, as **Pezeshk *et al.*, (2021)** studied the effect of using ultrasonication on the pH shift method, and it was found that it increased the solubility of the protein structure, although no differences appeared in the molecular weights in Sodium Dodecyl Sulfate – Polyacrylamide Gel Electophoresis (SDS-PAGE). Fats can be subjected to oxidation when using the pH shit method, especially in raw materials rich in hemoglobin **(Abdollahi *et al.*, 2020)**. Therefore, removing or reducing the fat content of raw materials gives protein concentrates with better qualities. This study aimed to reduce environmental pollution, take advantage of common carp (***Cyprinus carpio***) waste which include head, skin, scales, tail, and fins and use it in the preparation of protein concentrates as an alternative to expensive nitrogen sources in preparing culture media for the growth of lactic acid bacteria.

MATERIAL AND METHODS

Microorganisms

Lactobacillus rhamnosus GG,Lacto 66 , *Lactobacillus acidophilus*, Natures bounty, *Lactobacillus plantarum*, Swanson , *Bifidobacterium animalis* Bb12, Sandoz *Bifidobacterium infantis*, Biocodex.

Cultural medium

DeMan, Rogosa and Sharpe broth, Diagnostici Liofilchem, Italy.

Preparation of carp fish waste powder (*Cyprinus carpio*)

Live carp (***Cyprinus carpio***) were collected from local markets in Baghdad. The fish wastes which included the head, skin, scales, tail, and fins, were isolated, minced together using a meat grinderr, and dried in the oven at 40-50°C, for 5 h, to obtain the fish waste powder, then The powder was stored at -18°C until use.

Defatted

Following a methods previously describes by **(Rincon-Cervera *et al.*, 2017)** was used to remove fat from the waste of carp fish using a Soxhlet device. Hexane was used as a solvent, for 6 h, and the remaining solvent was removed, in the oven at 40°C, and grinding process was carried out with a grinder electrical, to obtain defatted powder of carp fish waste, and stored at - 18°C until use.

Proximate Composition

The moisture, protein and ash in carp waste and defatted fish waste powder were estimated according to the method described in **(AOAC, 2010)**, fat was estimated, according to the Folch method **(Saini *et al.*, 2021)**, carbohydrates was estimated according to the method described in **Mohammed, (2017)** from the following equation:

$$Carbohydrate\ \% = 100 - (Moisture + Protein + Ash + Lipid)$$

Determining the optimal pH for preparing protein concentrates

The aforementioned method was used by **(Akasha, 2014)** to determine the optimal pH for dissolution and precipitation of fish waste and defatted fish waste powder, by mixing them separately with 5% distilled water. The pH in the tubes was gradually adjusted from 1 to 14 using a hydroxide sodium solution 2N and hydrochloric acid solution 2N. The tubes were placed in a shaking water bath at room temperature for 60 min, followed by centrifugation at 6500 rpm for 15 min. The protein concentration in the liquid was estimated using the BradFord method using bovine serum albumin (BSA).

Preparation of protein concentrates

The method mentioned in **Yuanyong *et al.*, (2016)** was followed in preparing protein concentrate using the pH shifting method, by mixing carp waste, defatted fish waste powder with deionized water in a ratio of 1:9, using a magnetic stirrer. The pH was adjusted to 2, 12 using hydrochloric acid solution and sodium hydroxide 2N. The mixing process was completed using a magnetic stirrer for 60 min at 40°C, followed by centrifugation at 10,000 ×g, for 30 min, at 4°C. The precipitate was removed, acid supernatant (A.S) and base supernatant (B.S) fraction were collected, the pH was adjusted to 4.5 using a sodium hydroxide and hydrochloric acid solutions 2N, and the solutions was mixed using a magnetic stirrer for 60 min, at 40°C, followed by centrifugation 10,000 ×g, for 30 min at 4°C. The supernatant was removed and the precipitate were collected to obtain the acidic protein concentrates (ACO) and base protein concentrates (BCO), washed with deionized water, and the centrifugation process was repeated. The precipitate were collected, pH was adjusted to 7 and lyophilized.

Yield %

Estimate the yield of the acidic extract supernatant, the basic extract supernatant, the acidic protein concentrate, and the basic protein concentrate by the method mentioned in **Jaziri *et al*., (2020)** and as in the following equation:

$$Yield\% = \frac{Weight\ of\ extract\ or\ protein\ concentrate}{Weight\ of\ raw\ material} \times 100$$

Estimating the percentage of protein

The protein in each of the acidic and basic extracts and concentrates prepared from fish waste and defatted fish waste powder was estimated using the Kjeldahl method.

Preparation of alternative media

Alternative media were prepared by replacing Peptospecial and Beef extract in the MRS commercial medium with the acidic and basic protein concentrate, in the presence and absence of yeast extract, without changing the rest of the components of the commercial medium as in (Table 1). Media sterilized using autoclaves at 121°C for 15 min at 15 lb/in².

Table (1): Formulation of culture media at pH 6.2.

Ingredients (g / L)	MRS	Without yeast extract	With yeast extract
ACO , BCO	-	25	20
Peptospecial	10	-	-
Beef extract	10	-	-
Yeast extract	5	-	5
Glucose	20	20	20
Triammonium citrate	2	2	2
Sodium acetate	5	5	5
Magnesium sulphate	0.2	0.2	0.2
Manganese sulphate	0.05	0.05	0.05
Di – potassium phosphate	2	2	2
agar	15	15	15
Tween 80	1	1	1

Activation of bacteria

The MRS broth medium prepared by Diagnostici Liofilchem was prepared by dissolving 54.3g of the medium in 1000 mL of distilled water according to the supplied company's instructions, and mixing using a magnetic stirrer until a clear solution was obtained. The MRS medium was distributed in tightly sealed tubes and sterilized with an autoclave. After cooling to 37°C, the medium was inoculated with the contents of the lyophilized capsule of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus GG*, *Lactobacillus plantarum*, *Bifidobacterium infantis*, and *Bifidobacterium animalis* under sterile conditions, and incubated at 37°C for 72 h. Repeat the process three times, and store it in the refrigerator until use.

Preparation of MRS agar medium

DeMan, Rogosa and Sharpe (MRS) agar medium supplied by Diagnostici Liofilchem was prepared by dissolving 69.3g of the medium in 1000 mL of distilled water according to the supplied company's instructions, and mixed using a hot plate magnetic stirrer to completely dissolve the medium.

Bacterial growth in commercial and alternative media

The method mentioned in **Ahmed & Al - Shamary (2019)** was followed in preparing the decimal dilutions. The pouring plate method was used in growing the bacterial species, as 1 mL of each dilution was transferred to each type of bacteria into the Petri dishes, pour the commercial MRS agar medium and the alternative media, the plates were left to solidify, then they were transferred to the anaerobic incubation vessel, upside down, the air was removed, and incubated at 37°C for 48 h.

Statistical Analysis

All experiments were performed at least in triplicate. The results obtained were subjected to one-way analysis of variance (ANOVA). LSD Test was achieved to evaluate statistical significant difference ($p < 0.05$) using IBM SPSS 22.0 software.

RESULTS AND DISCUSSION

Proximate Composition

The result showed in (Figure 1) proximate composition of carp fish waste (FPB), which includes head, skin, scales, tail, and fins of carp fish and defatted carp fish waste powder (DFBPP). The moisture, protein, ash, fat, and carbohydrates in FPB were (67.4, 14.34, 5.7, 11.2, and 1.36)%, respectively, and in DFBPP were (5.6, 57.28, 25.23, 6.08, and 5.81)%, in same order. The drying process contributed to reducing the moisture in the waste of the fish from 67.4 to 5.6%. The defatting process also significantly reduced the fat to 6.08% in the defatted fish waste powder, which caused an increase in protein from 14.34 to 57.28%. **Jassim *et al.*, (2014)** found that the head of xylem tilapia fish contains (63.69, 16.07, 12.33, and 7.44)% moisture, protein, fat, and ash, respectively, while the skin and bones contain (62.11, 20.01, 15.35, and 2.27)%, in same order. **Rosmawati *et al.*, (2018)** studied the proximate composition of the skins and bones of snakehead fish (***Channa striata***), the skins contained (74.33, 18.49, 2.99 and 0.2)% each of moisture, protein, fat, and ash, respectively, while the bones contained (43.19, 15.49, 4.19, and 32.05)% in same order. **Maktouf *et al.*, (2020)** showed that the moisture, protein, fat, ash, and carbohydrates in ***Cyprinus carpio*** scales were (73.4, 22.1, 1.9, 3.89, and 1.38)%, respectively, in fins (55.8, 13.9, 3.6, 8.2 and 2.5)% in same order, and in bones (53.6, 25.9, 5.9, 1.48 and 1.9)% in same order.Studies indicate that variation in the proximate composition of fish is a result of different fish species **(Yousef & Al-Khshali, 2023)** fish farming systems used **(Stefka, 2016)** and fodder **(AL-khaaji & AL-Amary, 2016 ; Alrudainy& Jumaa , 2016; Mustafa & Al-Rudainy, 2021 ; Mohammad, 2023) .**

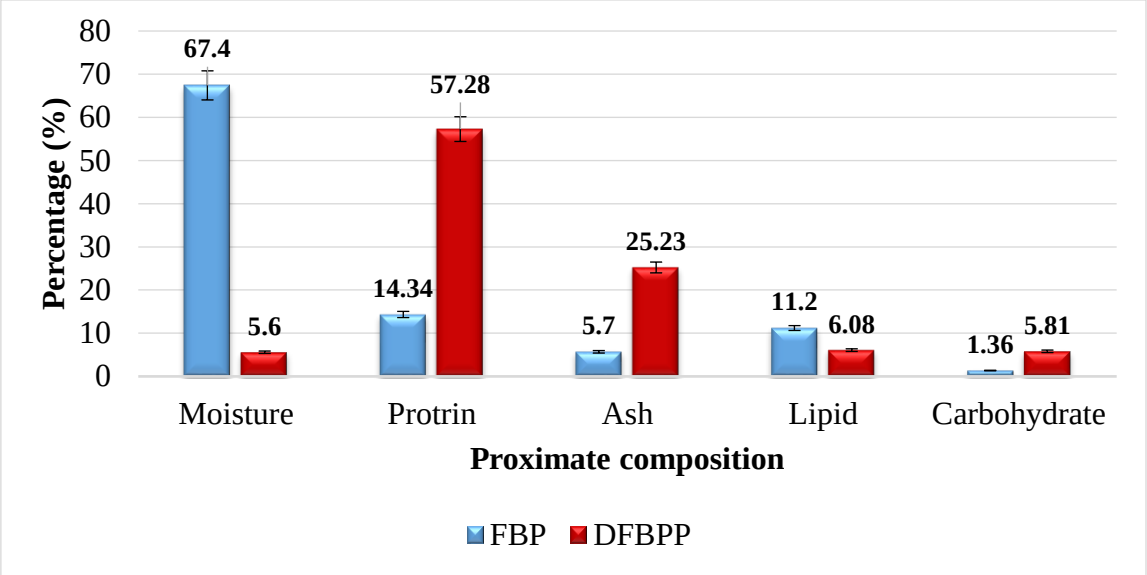

Figure (1): Proximate composition of carp fish waste and the defatted carp fish waste powder (FBP : carp fish waste , DFBPP : defatted carp fish waste powder).

Determine the optimal pH for dissolution and precipitation

(Figure 2) shows the optimal pH for dissolving and precipitating (FBP) and (DFBPP). Alkaline pHs gave the highest solubility, and the number 12 was the highest. Acidic pHs also gave high solubility, although the degree of solubility was high at pH 2, but it was lower than the solubility value at pH 12, while the lowest degree of solubility was at pH between 4 and 5 which was used in the preparation of protein concentrates, which represent the isoelectric point for a large percentage of fish waste proteins. Several factors affect colloidal solutions of proteins, such as the net charge, pH, ionic strength, and temperature **(Abood & Mohammed, 2017 ; Mohammad & Qasab Bashi, 2020)**. Proteins generally have a positive charge at pHs lower than their isoelectric point, and a negative charge at pHs higher than their isoelectric point, in both cases proteins spread in solutions and remain suspended due to repulsion between similar charges, and the resultant charge is zero at the isoelectric point, meaning that the total negative charge is equal to the total positive charge, as the different charges attract and proteins precipitate, and the number of negative charges increases whenever the pH is higher than the pH isoelectric point, due to the ionization of the alpha carboxylic groups belonging to the side chains of amino acids, while the number of positive charges increases as the pH decreases from the isoelectric point due to the ionization of the amine groups. This method has been used to prepare many protein concentrates from various animal and plant sources **(Al-Khshali& Al-Hilalli, 2017 ; Suhail *et al.*, 2022 ; Ali *et al.*, 2022)**. **Hyun *et al.*, (2016)** used this method to prepare protein concentrates from the roes of yellowfin tuna (***Thunnus albacares***) by adjusting the pH to 11and 12 to dissolve the, then precipitating them at 4.5 and 5.5. **Yuanyong *et al.*, (2016)** dissolved carp muscle proteins at pH 2.5 and 12.5 and precipitated them at 5.5, pH 5.5 represents the isoelectric point for myosin proteins, which have the highest percentage of muscle proteins. Fish waste contain collagen and elastin proteins, which are connective tissue proteins. Studies indicate the isoelectric point of collagen is

between 8 - 9 for the first type, which is called type A, while the second type has an isoelectric point at 4 - 5, which is called type B **(Garabawi *et al*., 2020 ; Kirdat & Dandge, 2021)**.

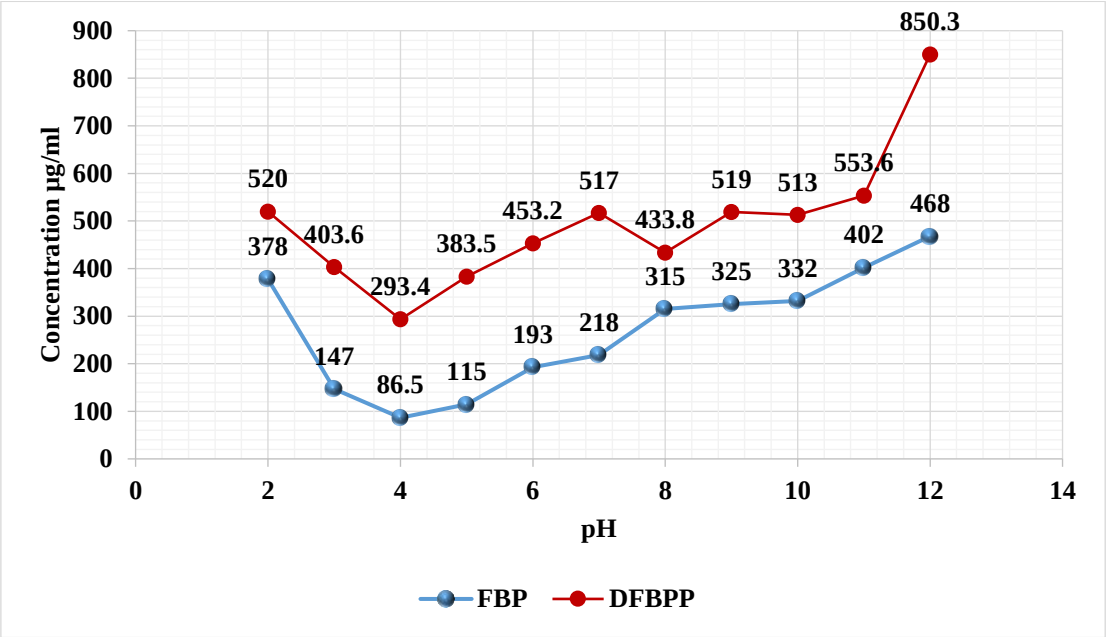

Figure (2): Determining the optimal pH for dissolving and precipitating carp fish waste and defatted waste powder. (FBP : fish waste , DFBPP : defatted waste powder)

Yield

(Figure 3) shows the yield of base supernatant (B.S), acid supernatant (A.S), base protein concentrates (BCO) and acidic protein concentrates (ACO) from carp fish waste, which amounted to (2.6, 2.4, 1.6, and 1.75)%, respectively and the defatted carp fish waste powder, which amounted to (1.22, 1.16, 0.67, and 0.63)% in same order. The process of removing fat significantly reduced the yield. The basic extract supernatant gave the highest yield because alkaline solutions help dissolve many substances and sonicate part of the fat, which is difficult to separate by centrifugation. The process of precipitation of proteins at the isoelectric point also reduced the yield because it precipitates only proteins and excludes dissolved substances. **Srikanya *et al.*, (2017)** prepared protein hydrolysates from heads and skeletons of Tilapia fish (*Oreochromis niloticus*) by Papain enzyme. The highest yield values were obtained at 70°C, pH 6.5 and enzyme concentration 1%, as the yield reached (7.3, 7.11, and 7.64)%, respectively. **Andreia *et al.*, (2021)** found that yield of protein hydrolysates prepared from the heads of Blue whiting, Red scorpionfish, Pouting, Gurnard, Megrim, and Atlantic horse mackerel by Alcalase (2.4 Anson Unit/g) was (87.7, 87.5, 90.4, 85.3 , 86.1and 90.3)%, respectively, and prepared from their skins and bones (97.3, 93.2, 95.7, 94.7, 90.2 and 88.3)%, in same order. **Nurdiani *et al.*, (2022)** prepared protein hydrolysates from heads of mackerel (*Scomber japonicus*), by incubating them at 30°C for (12, 24) h, at pH (5, 7, and 9), the yield reached (59.89 and 51.65)%. (49.77 and 35.98)% and (50.34 and 39.39)%, respectively.

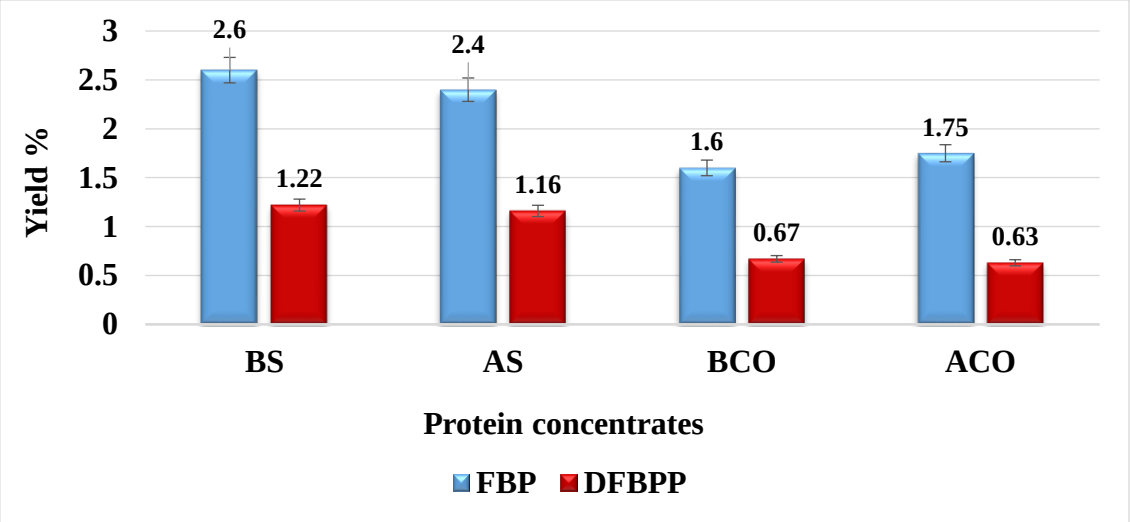

Figure (3): Yield of base supernatant (B.S) , acid supernatant (A.S) , base protein concentrates (BCO) and acidic protein concentrates (ACO) from carp fish waste (FBP) and defatted carp fish waste powder (DFBPP) .

Determination of protein in prepared extracts and concentrates

(Figure 4) shows the percentage of protein in each of basic extract supernatant at pH 12 and acidic extract supernatant at pH 2, basic protein concentrate and acidic protein concentrate, prepared from fish waste, which were (36.75, 34.65, 57.2, and 52.1)%, respectively, and those prepared from defatted fish waste powder (56.7, 49.7, 84.1, and 85.6)%, in same order. Process of removing fat from fish waste and precipitating proteins at the isoelectric point contributed to significantly increasing the protein content in the concentrates.**Ben Rebah *et al.*, (2008)** found that tuna waste extract supernatant prepared by thermal methods contained (10.26, 58.96, 26.79, and 3.99)% of each of fat, protein, ash, and carbohydrates, respectively, and sardine waste contained (35.5, 37.92, 20.6, and 5.96)% in same order. **Hyun *et al.*, (2016)** found that protein in isolates prepared from roes of yellowfin tuna (***Thunnus albacares***) using pH shift methods pH 11/4.5, pH11/5.5, pH12/4.5 and pH12/5.5 were (87.8, 83.2, 79.1 and 79.6)% respectively. **Haryati *et al.*, (2020)** reported when preparing a protein isolate from catfish, by dissolving and precipitating at pH 11 and 5.5, respectively, that the chemical composition of the isolate was (4.12, 1.05, 86.74, 0.54, 7.56)% for each moisture, Ash, protein, fat, and carbohydrates, respectively.

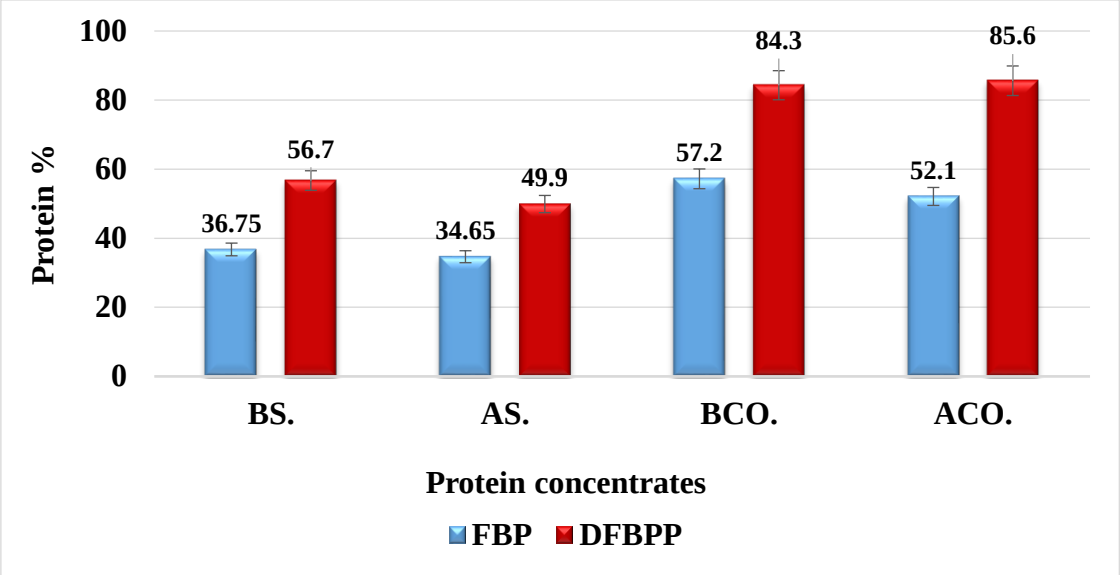

Figure (4): The Protein content of base supernatant (B.S) , acid supernatant (A.S) , base protein concentrates (BCO) and acidic protein concentrates (ACO) from carp fish waste (FBP) and defatted carp fish waste powder (DFBPP)

Growth of lactic acid bacteria on alternative culture media

(Table 2**)** show effect of (ACO) and (BCO) as a source of nitrogen instead of the nitrogen source in the MRS medium on growth of bacteria *Lactobacillus acidophilus*, *Lactobacillus rhamnosus GG*, *Lactobacillus plantarum*, *Bifidobacterium infantis*, and *Bifidobacterium animalis*. The number of colonies of *Lactobacillus acidophilus* bacteria in the alternative media containing basic protein concentrate was higher than in the media containing the acidic concentrate, adding yeast extract to both media significantly increased the number of colonies, but it was less than the commercial MRS media.The number of colonies of *Lactobacillus rhamnosus GG* bacteria was in the alternative media containing acidic and basic protein concentrates (90 and 102) × 10^5 respectively, the addition of yeast extract led to a significant increase in the number of colonies, but it was less than the number of colonies in the commercial MRS medium, in contrast to the *Lactobacillus plantarum* bacteria, which was given alternative media containing the acidic and basic protein concentrates in the presence of yeast extract, and basic protein concentrates in the presence of yeast extract produced a number of colonies equal to commercial MRS medium. The number of *Bifidobacterium infantis* colonies in the alternative media containing acidic and basic concentrates in the presence of yeast extract was higher than in the commercial MRS medium, in contrast to *Bifidobacterium animalis. Lactobacillus acidophilus* need many growth factors in addition to amino acids for growth **(Hassan & Saleh, 2016 ; Muhsin & Hassan, 2019 ; Kamran *et al.*, 2019)**. **Chen *et al.*, (2015)** showed that different nitrogen sources have an impact on the growth of *Lactobacillus acidophilus* bacteria, it was mentioned that organic nitrogen sources had higher growth than inorganic nitrogen sources, because they it contains free amino acids, peptides, glycosides, fats and growth factors **(Al-Lami *et al.*, 2015 ; Jasim & Fayyadh, 2018 ; AL-Shamary *et al.*, 2023)**. It has been found that complex nitrogen sources prepared from more than one type of peptone gave higher growth rates than yeast extract alone. . **Katarzyna &**

Agnieszka (2020) found when growing, *Lb. rhamnosus ŁOCK 1087* and *Lb. plantarum ŁOCK 0860* on cultural media containing wheat, barley, corn, and rye, as the growth rate of bacteria was (0.21 and 0.22) 1/h, respectively, and in MRS medium (0.12 and 0.15) 1/h, as found by **Mengyu *et al.*, (2024)** showed that the bacteria *L. plantarum* and *L. rhamnosus* have the ability to use different types of nitrogen sources, he also indicated that they gave high growth rates when using culture media containing peptides with high molecular weights compared to the bacteria *L. reuteri* and *L. fermentum*.

Table (2): Effect of the basic concentrate (BCO) and the acidic concentrate (ACO) as a source of nitrogen instead of the nitrogen source in MRS medium on the growth of lactic acid bacteria.

Lactic acid bacteria cfu/ mL	MRS medium	Without yeast extract		With yeast extract	
		BCO	ACO	BCO	ACO
Lactobacillus acidophilus	290×10^5 a	102×10^5 b	90×10^5 c	183×10^5 d	170×10^5 e
Lactobacillus rhamnosus GG	148×10^6 a	122×10^6 b	130×10^6 c	138×10^6 d	140×10^6 d
Lactobacillus plantarum	225×10^8 a	226×10^8 b	221×10^8 a	225×10^8 a	225×10^8 a
Bifidobacterium infantis	199×10^8 a	180×10^8 b	212×10^8 a	202×10^8 a	217×10^8 c
Bifidobacterium animalis	134×10^4 a	38×10^4 b	42×10^4 bc	45×10^4 bc	46×10^4 bc

Note: Means with different letters in each row are significantly different ($p < 0.05$).

CONCLUSION

In this research, we reached the possibility of preparing protein concentrates from common carp fish waste using a pH shift method, as pH 2 and 12 gave the highest solubility for proteins. The process of removing fat from fish waste and precipitating proteins at pH 4.5 contributed to the increase in protein in the protein concentrates. Protein concentrates prepared from defatted powdered carp fish waste can be used to prepare alternative culture media at low cost for the growth of lactic acid bacteria.

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