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Optimization of Enzyme Assisted Alkaline Extraction of Sunflower (Helianthus Annuus L.) Protein for Alternative Isolate Production

Dmitry V. Baurin, Alexandra V. Baurina*, Irina V. Shakir, Victor I. Panfilov

Department of Biotechnology, Mendeleev University of Chemical Technology of Russia, Miusskaya sq. 9, 125047 Moscow, Russia
tur.alexandra96@gmail.com

This research focused on the effect of extraction time (60–120 min), enzyme/substrate ratio (0.05–0.4 %) temperature (37.5°C) on enzyme assisted alkaline protein extraction efficiency from non-dehulled sunflower meal, which is a by-product of oil production. 4 commercial enzymes were studied and the highest protein yield (92 %) was obtained by ultrafiltration of the enzymatic hydrolysates through a membrane (selectivity 81.4%) followed by the isoelectric precipitation (pH 4.5) and vacuum drying. The protein yield was found 92.85% at the optimized condition of enzyme assisted alkaline extraction: temperature 37.5 °C, 60 min, and enzyme/substrate ratio of 0.2%. Compared to alkaline extraction with 0.25% NaOH and no enzyme addition, the protein yield has increased by 31% due to the mild extraction conditions. Determined physicochemical properties of the obtained protein isolate: high protein content (92.85% d.b) as well as high solubility (95.0% at pH 7.5) reflect the unique characteristics of the product. However, the process requires further optimization because of the green colour of the isolate, which limits its application in the food industry.

1. Introduction

The problem of food protein deficiency increasing around the world demands research of additional food resources, the actual solution being the use of vegetable proteins for creating products of increased nutrition and biological value. Production of high nourishing foodstuff has to be expedient.

The development of low-waste effective technologies meeting the requirements of ecological safety and decrease in power consumption is of important economic value. The particular interest is caused by meals which are formed in the course of oil-bearing crops seeds processing, as potential raw materials for increasing protein value of meat processing and bread baking products (Sharma, A. et al., 2001).

Sunflower is one of the most promising crops to isolate the protein. European countries, Ukraine and The Russian Federation are the biggest producers of sunflower seeds in the world and the most interested in its full use and processing.

The European Commission and member states have pointed at advantages of a more balanced supply and consumption of domestic protein crops as part of an integrated strategy responding to new challenges like climate change, agricultural biodiversity loss, depletion of soils, pollution of groundwater, and price volatility for agricultural products on the world market (EC, 2021). Today the sunflower meal (SFM) which is formed at oil extraction from sunflower seeds is underestimated as a food protein source. Normally SFM can be included in the diets of pigs, poultry, cattle, and horses of all ages (Lomascolo, A. et al., 2012). Its main advantage is as a good source of linoleic acid. The inclusion rate can be up to 10% according to suppliers. Sunflower protein surpasses many crops on the content of essential amino acids and it is the second only to soybean seed protein content of lysine, but it differs more high digestibility and absence of enzyme inhibitors of trypsin (Shchekoldina, T., & Aider, M., 2014). Integrated processing is to provide isolates and protein hydrolysates, which can be used in food production to increase the protein value of processed meat and bakery, as well as fodder production and microbiological synthesis.

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The depth of processing, quality of the final products, and reduction of waste are important aspects of emerging technologies (Salgado, P. R., et al., 2012). Compliance with the principles of green technologies and strategic evaluation of an economic effect is underpinned by the technology being developed.
In the current economic situation, the Russian Federation exports SFM with high protein content. However it is shown that the use of sunflower protein can compensate for the lack of protein in food (Bautista, J. et al., 2000), and biological conversion of SFM processing products allows producing feed additives enriched with microbial protein (Bautista, J. et al., 1990). Research in the area is carried out in many countries and various options for recycling this valuable secondary product into the feed and food concentrates are offered. Studies are dedicated to the chemical, and enzymatic treatment of the meal, isolation and purification of proteins, and utilization of solid deproteinised meal.
Chemical extraction techniques such as extraction with saline (Shchekoldina, T., & Aider, M., 2014) and alkaline solutions (González-Pérez, S., & Vereijken, J. M., 2007, Soo M.H., et al., 2021) as well as acid extraction (Pickardt, C., et al, 2009, 2011; Weisz, G. M., et al, 2010) were proposed to isolate the protein. Protein precipitation from solutions was carried out either with organic solvents, or at the isoelectric point using succinic acid and others (Ordonez, C. et al., 2001). All the methods mentioned have several disadvantages, for example, the alkaline treatment causes binding of phenolic compounds, such as chlorogenic acid to amino and thiol groups of amino acids (Pickardt, C. et al., 2009), which in turn leads to the formation of dark-colored products. The use of enzymes allows deproteinising plant materials under mild conditions (Sari, Y. W. et al., 2013), significantly reducing the amount of alkali introduced into the process, and the use of energy-efficient processes for the purification of the isolates can greatly reduce the cost of obtaining the final product (Salgado, P. R., et al., 2012).
The extraction of proteins from sunflower meal can be carried out both at acidic and alkaline pH values. The determining criterion for evaluating the efficiency of extraction was the protein yield from the maximum possible in terms of the content of crude protein in the meal. From the analysis of literature data, extraction methods such as extraction with concentrated salt solutions, dilute solutions of acids, and alkalis were identified. The most promising from the point of view of the technology for obtaining food isolates is extraction with solutions of sodium chloride and hydroxide. The use of concentrated salt solutions for extraction is associated with several technological difficulties and should include laborious stages of purification from sodium ions. In this paper, we propose a processing option that does not have this drawback.
This work aims investigate the possible use of enzymes in assisting the extraction of protein from SFM under mild conditions and the selection of the most efficient enzymatic proteolytic complex. Ultrafiltration for the concentrating and removal of low molecular weight protein fraction and carbohydrates from crude sunflower extracts was developed in the present study. The comparative analysis was carried out and basic parameters such as productivity rate, the maximum extent of concentration, general losses of protein in relation to the initial solution were determined.

2. Materials and Methods

2.1 Materials and chemicals

Industrially defatted with hexane non-dehulled sunflower meal (SFM) was purchased from “EFCO” (Belgorod, Russia). Chemicals (analytical grade) were purchased from Sigma Aldrich.

2.2 Enzymatic hydrolysis

Four commercial enzymes Protex 7L, Protex 40E, Protex 6L, and Protex 51FP were obtained from Genencor (Danisco International Moscow, Russia). All the proteases were alkaline and food-grade with optimum pH range from 7.0 to 12.0, which corresponds with the solubility of sunflower proteins. Protex 40E is a *Bacillus subtilis* alkaline protease; Protex 51FP - endo/exo-peptidase obtained with *Aspergillus oryzae;* Protex 6L - *Bacillus licheniformis* alkaline serineprotease; Protex 7L - *Bacillus amylo-liquefaciens* alkaline endopeptidase. Enzymatic hydrolysis parameters are presented at Table 1.

Table 1: Enzymatic hydrolysis parameters

Parameters	Protex 40E	Protex 51FP	Protex 6L	Protex 7L
pH	8.6	8.5	9.5	7.1
Temperature (◦C)	37	37	37	37

Sunflower meal was poured with water in a ratio of 10:1, an enzyme preparation was added and placed in a thermostat with continuous mixing. The enzyme concentration varied from 0.05% to 0.4% by weight of the

meal. The duration of hydrolysis did not exceed 120 min, the temperature of hydrolysis was 37°C. Each series of experiments was carried out three times; the arithmetic mean was taken as the final result.

2.3 Ultrafiltration

The obtained during the enzyme-assisted alkaline extraction hydrolysates were concentrated using polysulfonamide membrane UPM-20. The ultrafiltration module consisted of the cell, magnetic stirrer, compressor, manometer, valve; permeate line, gear drive.

2.4 Isoelectric precipitation of the hydrolysates

Isoelectric precipitation from the hydrolysates was carried out by adding acid solutions, volume 10 ml for 10 min with constant stirring on an orbital shaker (180 rpm). To achieve complete precipitation, the solutions were cooled at 4–6°C. The required pH value was established with HCl and NaOH. The formed precipitate was separated by centrifugation. The protein content was determined in the supernatant and sediment.

2.5 Drying

The obtained sunflower protein enzymatic hydrolysates and isolates were dried on a laboratory spray dryer and a vacuum dryer. Spray drying of the hydrolysate was carried out on a laboratory Mini Spray Dryer B-290, Büchi, with a maximum water capacity of 1 kg/h. The temperature of the drying agent at the inlet to the apparatus was varied from 180°C to 200°C, depending on the selected mode. The flow rate of the hydrolyzate was chosen so that the temperature of the drying agent at the exit from the drying chamber did not exceed 80±2 °C during the entire process. Vacuum drying was carried out on a CoolSafe 100-9 unit without preliminary freezing of the material.

2.6 Chemical analysis

Crude protein, crude fiber, carbohydrates, and moisture content were determined on a dry matter basis (George W., Latimer, J. R., 2019). The extraction yields were expressed as percentages of the component in each fraction relative to the initial amounts in the material. Deproteinisation was held under alkaline conditions to provide higher protein solubility. The amount of extracted protein varies from 19 to 65%, depending on the pH of the extract (amount of NaOH) (González-Pérez, S., & Vereijken, J. M., 2007). Proteinogenic amino acids content was determined by capillary electrophoresis using the Kapel-105 system (wavelength 254 nm, temperature 30 °C, pressure 30 mbar, input time 5s, voltage 25 kV, pressure 0-50 mbar, analysis time 10-15 min).

3. Results and discussion

The isoelectric point of sunflower protein is 4.5. Compared to the acidic conditions more protein was extracted from selected biomass under the alkaline conditions. Alkaline protein extraction allows to isolate a significant amount of protein. Up to 65% of the protein compounds were extracted during the alkaline extraction with NaOH solution at a pH>9.5. However, the resulting product must be purified. Oxidation of phenolic compounds and chlorogenic acid at alkaline conditions form the green color of the protein isolate along with the present husk pigments. Consequently, the resulting extract has lower quality and is not suitable for the food industry.

To obtain light-colored solutions under mild conditions the enzyme assisted protein extraction was applied. To determine the optimal conditions for enzyme assisted alkaline extraction of SFM the effects of enzyme preparations Protex 7L, Protex 40E, Protex 51FP, and Protex 6L were studied.

More than 50% of biomass protein was extracted after 2 hours of hydrolysis at 37°C and adjusted pH with Protex 7L, Protex 40E, and Protex 6L. These three enzymes gave comparable protein recovery. The use of Protex 51FP at pH 8.5 did not result in sufficient protein extraction yield and the use of this enzyme appeared inexpedient. Solid waste obtained after protein extraction - deproteinized meal, contained less than 25% of protein and more than 38% of crude fiber when treated with Protex 40E. This product is a promising medium for solid-state fermentation. The liquid extracts were light-colored and did not appear to change color without extra treatment. Extract, obtained with Protex 40E (dry weight 4.26%), was chosen for further investigation since no additional amount of alkali was required to obtain the required quality.

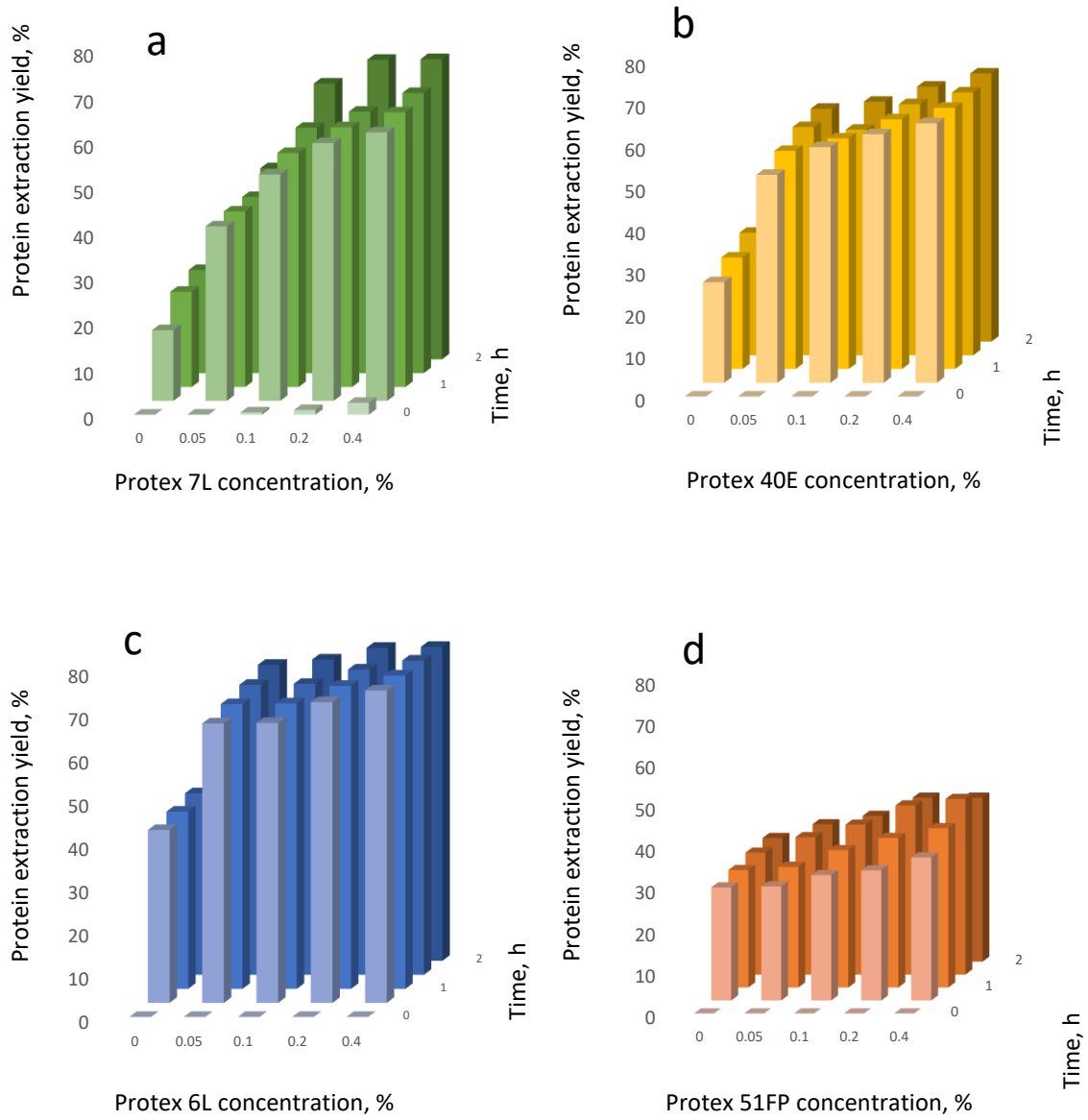

Figure 2: Enzyme assisted alkaline extraction of sunflower protein with Protex 7L (a), Protex 40E (b), Protex 6L (c) and Protex 51FP (d) at variable enzyme concentrations 0, 0.05, 0.1, 0.2 and 0.4 %, respectively).

Different membranes were studied to remove low molecular weight proteins and carbohydrates from the extract to obtain protein isolate. The concentration was studied using UPM-20 ultrafiltration membrane. The basic parameters of the process were membrane performance 10±1 L/m^2/h and degree of concentration - 10. The main product parameters of the protein extracts after spray drying are presented in Table 2. The products were flowable fine powders with light grey color.

Table 2: Crude protein, product yield, moisture, carbohydrates content in sunflower protein isolate sample (%), obtained with Protex 40E after spray drying at 180 and 200 ^{o}C, respectively

№	Crude protein	Product yield	Moisture, w/w	Carbohydrates
1	92.8 ± 0.5	90.5± 0.3	3.8 ± 0.2	7.0 ± 0.2
2	92.8 ± 0.5	52.0 ± 0.3	4.6 ± 0.2	3.0 ± 0.2

Sample 2 was dried at a higher temperature, thus having a higher moisture content and had darker color. High temperature resulted in sugar caking, which formed a crust on the surface and prevented evaporation. Sticky particles of the sample were stuck in the chamber and lowered the product yield. The crude protein content of the obtained sunflower protein isolate reached 92.85%.

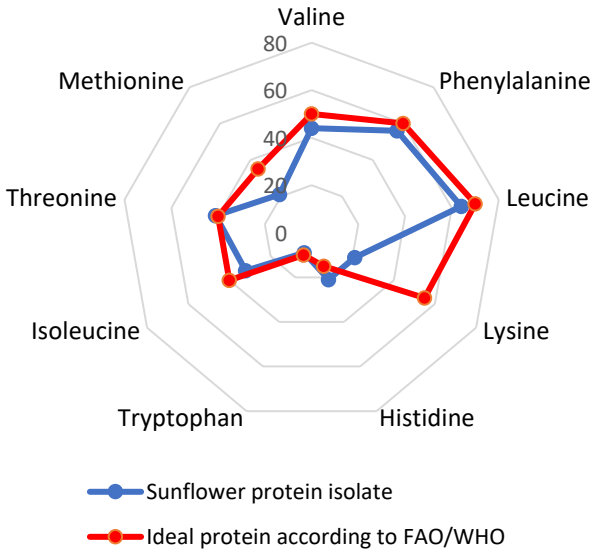

Figure 3: Amino acid composition of sunflower protein isolate, compared to ideal protein according to FAO/WHO, mg/g protein

The amino acid score is an indicator of the biological value of a protein, which is the percentage of the consumption of essential amino acids to the standard (recommended) use of this proportion. Data on the amino acid score of the obtained sunflower protein isolate and ideal protein (WHO) are presented in Figure 3.

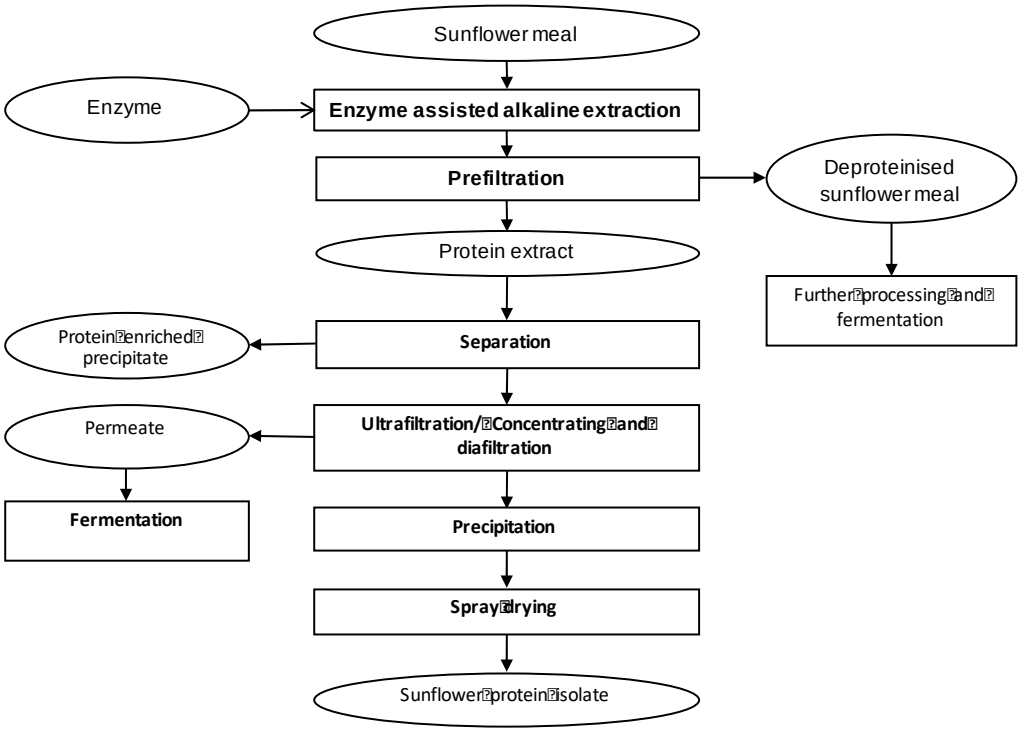

Figure 4: Basic technological scheme for the production of sunflower protein isolate

A basic technological scheme of enzyme assisted alkaline extraction of protein from non-dehulled sunflower meal was performed (Figure 4) based on the obtained experimental results. The technological process includes enzyme assisted alkaline extraction, filtration, separation, ultrafiltration (concentrating), and spray drying.

4. Conclusions

It has been shown that the use of commercial proteolytic enzymes can intensify the alkaline extraction of sunflower protein isolate under mild conditions. The extraction of protein compounds from sunflower meal increased up to 72.5%

The technology of obtaining sunflower protein isolate with crude protein content 92.85% includes enzymatic treatment of sunflower meal, ultrafiltration, precipitation from the concentrate at the isoelectric point, followed by washing and vacuum drying. By-products of the extraction process, such as permeate and deproteinised sunflower meal, can be a good source of nutrients for microbiological fermentation.

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