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5 Applications of polysaccharidases for cold pressing of seeds

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Abstract

The aim was to develop an efficient method to extract oil from low oil producing crops (tomato and pomegranate seeds) by cold pressing, without losing its nutritional qualities. The work included 5 main tasks.

Task 1. *Isolation and identification of main components from tomato and pomegranate seed shells, which contained 15.3 and 18.7% of cellulose, 11.5 and*

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21.4% of lignin, 7.9 and 13.1% of readily hydrolysable polysaccharides (RHP) and 20.0 and 31.4% non-readily hydrolysable polysaccharides (NRHP), respectively.

Task 2. *Screening and selection of fungi producing high levels of plant cell-wall degrading enzymes. Cellulases I and II were purified and characterized from Aspergillus terreus. An endoglucanase and a xylanase from a psychrophilic fungus, Aspergillus foetidus; a xylanase and a polygalacturonase from a psychrophilic fungus Cladosporium herbarum were purified and characterized.*

Task 3. *Purification and characterization of lignin-peroxidase from P. ostreatus.*

Task 4. *The determination of the effect of cell wall degrading enzymes on carbohydrate part of seed shells and the amount required. Enzymes from A. terreus (cellulases I and II), A. foetidus (endoglucanase and xylanase), C. herbarum (xylanase and Polygalacturonase) and P. ostreatus (lignin peroxidase) at a ratio of 1:2:1:3 at pH 4.0 and 40^0C were suitable for optimal treatment of the lignocarbohydrate part of tomato and pomegranate seeds. The best combination of enzymes increased the degree of swelling, release of reducing sugars, weight loss, and disintegration of the structure of tomato and pomegranate seed shells. Also, this process was monitored by electron microscopy.*

Task 5. *The humidity control of seeds before pressing, the temperature of seeds, oil, oil cake and press forming matrix as well as the pressure through electronic motor, the optimization of conditions for extracting oil from seeds by cold pressing. Using the mini-press, oil from tomato and pomegranate seeds with 8-10% humidity was extracted by cold-pressing at temperatures below 40^0C. The mechanical grinding of seeds revealed that a particle size of 250 μm was optimal to extract maximum oil during cold pressing. The treatment of tomato and pomegranate seeds with plant cell wall degrading enzymes followed by cold pressing increased the oil yield by 34.5 and 66.7%, respectively.*

The tomato and pomegranate seed oils extracted by cold pressing after enzyme treatment contained high levels of micronutrients than the oils extracted using chemical extraction method. Pomegranate seed oil contained 26.1 and 155 mg/g vitamins A and E, while tomato seed oil contained only 1.5 mg/g vitamin A. In contrast, the oil cake from both seeds contained appreciable amounts of vitamins A, C, E, B_1, B_3, B_6 and Pyridoxal/Pyridoxamine phosphate (PP). Pomegranate oil cake was rich in vitamin B_3 (0.5g/100g). Also, it was shown that the above two oils were rich in unsaturated fatty-acids.

In conclusion, the enzyme treatment improved the quality of oil with respect to micronutrients, including vitamins. The results would provide novel information on cold pressing, enzyme treatment and on the valuable properties of tomato and pomegranate seeds oils. Also, these new results will pave the way for further research and commercialization of this novel method and marketing of nutritionally high value oils.

1. Introduction

Currently, hot and cold pressing as well as chemical extraction methods are used for the extraction of oil from seeds [1]. Among these, hot pressing and chemical extraction methods are well adapted for the extraction of oil from soyabean, peanut, palm, cottonseed, sunflower and other plants.

Besides the well-known oil-producing seeds, there are seeds containing low oil with valuable biological properties. Two such seeds are from tomato and pomegranate. Their oil is rich in microelements, vitamins A, D, E and F and has antioxidant [2-5], anti-tumor [6-9] and anti-phlogistic [10-14] properties. Thus, these can be used in pharmacology, cosmetology and in food industries as valuable nutritious components.

For the extraction of oil from low oil producing seeds, it is undesirable to use hot pressing and chemical extraction methods. It is likely that the high temperature would destroy the natural composition, produce unwanted byproducts and lead to the loss of valuable biologically active substances. In case of chemical extraction method, the use of organic solvents and their residue could influence the value of final product. In contrast, the cold pressing could fully retain the natural composition and biological value of the oil obtained. However, the cold pressing of low oil producing seeds is not ideal since a large amount of extracted oil is usually absorbed into the oil cake. This is mainly due to the polysaccharide part of seed shells. Also, the preliminary seed hulling before pressing decreases the oil yield due to the loss of the pulp mass during mechanical removal of the seed shells. Therefore, cold pressing is unprofitable for extracting oil from seeds with low content.

It is well known that the main components of seed shells are cellulose, hemicellulose, lignin and tannin [15]. The ratio of these components varies with the type of seeds and their origin. It is interesting to evaluate the role of cell wall degrading enzymes on cold pressing of low oil producing seeds in order to extract their oil in a profitable manner. Many fungi and bacteria are known to produce cellulases, hemicellulases, pectinases, ligninases, and tanninases to degrade the plant cell wall. Therefore, few micro-organisms were chosen to produce the above enzymes, which were used to partially hydrolyse the seed cell wall. This would be an interesting way forward for increasing the oil yield from seeds with low oil content.

2. Materials and methods

2.1. Analysis of lingo-carbohydrate structure of the tomato and pomegranate seed shells

2.1.1. Seed processing

A 100 g of dried tomato/pomegranate seeds were homogenized and preliminary de-oiling carried out using 3 volumes of hexane and chloroform.

This was placed in a "Soxlet" apparatus and extracted using 200 ml ethanol and benzene mixture (1:2) for 24 h (3 times, each for 8 h) [16]. The seeds were dried and powdered using a grinding mill.

2.1.2. Isolation of sulfur-acidic lignins (Komarov lignins)

For this purpose, the Komarov method was used [16]. The tomato/pomegranate seed powder (1g) was placed in a round-bottomed flask and processed by adding 15 ml of 72% H_2SO_4 for 2.5 h at 25^0C. The mixture was diluted to 200 ml with distilled water and boiled for 1 h. The reaction mixture was filtered through glass filter No. 1 and the residue obtained after neutralization was dried. Products obtained were calculated as a %, of the initial seed weight.

2.1.3. Isolation of hydro-chloro-acidic lignins (Wilshtetter lignins)

These were isolated as described [17]. A 50 g tomato/pomegranate seed powder was placed in a round-bottomed flask and added with 480 ml HCl (12.1 M), and the suspension was stirred for 35 min at room temperature. The residue was separated and the filtrate was added with 218 ml water and kept over night. The precipitate formed was filtered, washed and suspended in 1.3L of 0.1 M H_2SO_4 and boiled for 5-6 h. The precipitate was then filtered, neutralized with NaOH and dried at room temperature. The products obtained were calculated as a % of the initial seed weight.

2.1.4. Gel filtration of Wilshtetter lignin

Gel filtration of hydro-chloro-acidic lignin was carried out on a Sephadex G-75 (1.6x100 cm) column, using di-methylsulfoxide (DMSO) as a solvent. Molecular mass distribution was determined as described [18]. Elution was carried out at a flow rate of 30 ml/h with 1.0-1.5 ml fractions. The lignin was detected at 280 nm with the sensitivity of 0.1.

2.1.5. De-lignification and cellulose isolation

De-lignification and cellulose isolation were carried out as described [16]. A 1 g tomato/pomegranate seed powder was placed in a 250 ml round-bottomed flask with 25 ml mixture of HNO_3 (15.8 M) and ethanol (1:4) and boiled for 1 h. The reaction mixture was filtered through glass filter and the residue formed was isolated and placed again in a round-bottomed flask and the above procedure was repeated until the end of de-lignification. The de-lignification was confirmed by the disappearance of red colour, when phloroglucinol was added to the solution [16]. The cellulose obtained was calculated as a % of the initial seed weight.

2.1.6. Determination of readily hydrolysable polysaccharides (RHP) and non-readily hydrolysable polysaccharides (NRHP)

RHP and NRHP were determined at different conditions as described [17]. A 5 g of cellulose was placed in a 500 ml round-bottomed flask with 200 ml of 0.2 M HCl and the reaction mixture was boiled for 3 h and filtered before neutralizing the residue using water. The filtrate was washed with water and diluted to 500 ml. The content of RHP in the solution was determined using the following formula:

$$X = \frac{Cp * V * Kp}{g}$$

g – the weight of absolute dried seeds; C_p – concentration of reduced substances in RHP, %; V – volume of hydrolysate, ml (500 ml); Kp – coefficient for re-calculation of monosaccharides to the polysaccharides, equal to 0.89.

During the second step, the dried residue was placed in 1L round-bottomed flask and stirred with 40 ml of 14.6 M H_2SO_4 at room temperature for 3 h. The reaction mixture was diluted with 600 ml distilled water and boiled for 5 hrs before filtering and washing with water. The content of the NRHP in the filtrate was determined using the following formula:

$$X = \frac{Ct * V * n * Kt}{g}$$

g – the absolute weight of dried residue; C_p – concentration of reduced substances in NRHP, %; V – volume of hydrolysate, ml (1000 ml); Kp – coefficient for re-calculation of monosaccharides to the polysaccharides, equal to 0.89. n – Dilution of hydrolysate at neutralization.

2.1.7. Functional group analysis

Analysis of OH, CO and COOH functional groups in tomato and pomegranate seed shell lignin was performed [19].

a) Analysis of total OH groups

A 20 mg tomato/pomegranate lignin was mixed with 1 ml pyridine and acetic acid (4.4 M) at a ratio of 4:4.7 and incubated at 50^0C for 24 hrs. The reaction mixture was titrated by potentiometric method. Amount of total OH groups was calculated using the following formula:

$$OH = \frac{a * b_0 * f * 170}{(a_0 - b) * A}$$

A – weight of lignin, mg; a – weight of acetylation mix, mg; a_o – weight of acetylation mix in the blank, mg; b – volume of 0.1 N NaOH, which was spent

for titration, ml; b_o – volume of 0.1 N NaOH, which was spent for blank titration, ml; f – factor to the titration of 0.1 N NaOH.

b) Analysis of CO groups

A 20 mg tomato/pomegranate lignin was dissolved in 20 ml ethanol before adjusting the pH to 4.0 and incubated in a titration cell at 25^0C for 24 hrs. A 138 mg (2mM) of NH_2OH*HCl dissolved in 40 ml of 80% ethanol was added and titrated with 0.01 M NaOH. The amount of CO groups in lignin was calculated using the following formula:

$$CO = \frac{a * f * 0.2801}{A} * 100$$

a – volume of 0.01 M NaOH which was spent for titration, ml; f – factor to the titration of 0.01 M NaOH; 0.2801 – the mass of CO group which was equivalent for 1 ml of 0.01 M NaOH, mg; A – weight of lignin, mg.

c) Analysis of COOH groups

A 40 mg lignin was placed in a 25 ml volumetric flask and treated with 20 ml of 0.12 M $(CH_3COO)_2Ca$ at 85^0C for 30 min. After cooling, the reaction mixture was dissolved in 25 ml distilled water and filtered. A 20 ml filtrate was titrated against 0.05 M NaOH using phenolphthalein as an indicator. The amount of COOH groups was calculated using the formula below:

$$COOH = \frac{(a - a_0) * f * 1.25 * 0.85}{A} * 100$$

a and a_o – volume of 0.05 M NaOH used for titration in ml; f – factor to the titration of 0.05 M NaOH; 0.85 – the mass of OH groups in COOH which were equivalent to 1 ml of 0.05 M NaOH, mg; 1.25 – coefficient for re-calculation of the total volume; A – weight of lignin, mg.

2.1.8. Infra-Red (IR) analysis

IR spectra of samples treated with potassium bromide were obtained using a Fourier spectrophotometer (model “Perkin – Elmer” 2000).

2.1.9. Alkaline hydrolysis

A 5 g tomato/pomegranate seed powder was processed with a 100 ml of 2 M NaOH and boiled at 100^0C for 1 h. The reaction mixture was filtered through a glass filter and acidified using concentrated H_2SO_4 by adjusting the pH to 1.0. The lingo-carbohydrate precipitate (suspension particles) was washed and kept for further analyses. The hydrolysate obtained was extracted

by ether and treated with a saturated solution of $NaHCO_3$ to obtain the phenol acidic fraction. The phenol acid content was determined by using HPLC.

2.1.10. Acidic hydrolysis

Acidic hydrolysis of suspension particles was carried out as described [20]. A 0.1 g suspension dissolved in 4.0 ml of 2N H_2SO_4 was boiled for 48 hrs. The hydrolysate was centrifuged and the supernatant concentrated to 1 ml. A 0.5 ml sample was mixed with 0.5 ml of 0.5% phenol and 2.5 ml concentrated H_2SO_4. The intensity of the brown colour was measured at 280 nm using the spectrophotometer.

2.2. Isolation of cellulase from *Aspergillus terreus*

2.2.1. Chemicals

Sephadex G-10, TSK HW-55f and DEAE-TSK HW-650S were from Pharmacia, Sweden and Toyo Soda Company, Japan, respectively. SDS-PAGE was performed as described [21]. *Aspergillus terreus* was from the Institute of Microbiology Academy of Sciences Republic of Uzbekistan. All the remaining chemicals were AnalaR grade and from Sigma.

2.2.2. Growth conditions

Aspergillus terreus was cultured using a medium containing agricultural byproducts such as press cake from seeds of cotton plant/birch rasps at different concentrations. Cultivation was carried out using 500 ml Erlenmeyer flask at 240 rpm up to 17 days at 30^0C.

2.2.3. Endoglucanase activity

Endoglucanase activity was determined as described [22]. A 0.1 ml culture filtrate or enzyme sample was added to 1 ml of 1% CM-cellulose and incubated at 40^0C for 30 min. A 2 ml solution consisting of 4 volumes of Somogyi reagent I and 1 volume of Somogyi reagent II (mixed immediately before use) was pipetted to a 10-ml tube along with a solution containing reducing sugar (5-200 μg) and water to give a total volume of 4 ml. The mixture was boiled in a water bath for 15 min, cooled and a 2 ml of Nelson reagent was added before mixing using a Vortex. Finally, 4 ml of distilled water was added to the solution and mixed by inversion. The absorbance was read at 520 nm and translated (after subtraction of the absorbance of reagent blank) into glucose equivalent using a standard graph obtained by plotting micrograms glucose Vs absorbance.

2.2.4. Ammonium sulphate precipitation and desalting

The protein was precipitated with 3 M $(NH_4)_2SO_4$ and desalted on a Sephadex G-10 column (2.6x50 cm) at a flow rate of 30 ml/h using distilled water. Protein was detected at 280 nm with a sensitivity of 0.1.

2.2.5. Gel filtration

A 152 mg freeze-dried sample was dissolved in 5 ml 0.01M sodium acetate buffer, pH 4.9 and applied on to a TSK HW-55f column (1.6x125 cm), equilibrated with the same buffer and eluted at a flow rate of 30 ml/h. The protein detection was carried out at 280 nm with a sensitivity of 0.1.

2.2.6. Ion exchange chromatography

The active fractions (340 ml) from the above step were applied on to a DEAE-TSK HW-650S column (1.6x15 cm), equilibrated with 0.01 M sodium acetate buffer, pH 4.9. The column was washed with the above buffer and eluted with 500 ml 0.0-1.0 M sodium chloride prepared in the same buffer, at a flow rate of 60 ml/h. The protein detection was carried out at 280 nm with a sensitivity of 0.1.

2.2.7. Protein determination

Protein was determined by the method of Lowry et al. [23] using bovine serum albumin as a standard.

2.2.8. SDS PAGE

This was performed as described [21].

2.2.9. Determination of pH and temperature optima

The temperature optimum was determined by carrying out the standard assays with varying temperatures between 20 and 70^0C in 0.05 M sodium acetate buffer, pH 4.5. The optimum pH was determined by measuring the activity at 40^0C over a pH region between 3.0 and 7.0 using 0.05 M sodium acetate and 0.05 M sodium phosphate buffers at the pH regions between 3.0-6.0 and 6.0-7.0, respectively.

2.3. Isolation of polysaccharidases from *Aspergillus foetidus* and *Cladosporium herbarum*

2.3.1. Chemicals

The Bio-Gel P-6 was from Pharmacia, Sweden. *Aspergillus foetidus* and *Cladosporium herbarum* were from the IMI UK. All the remaining chemicals were AnalaR grade and from Sigma.

2.3.2. Growth conditions

Aspergillus foetidus and *Cladosporium herbarum* (IMI 385292) were maintained on a potato agarose gel. 1 L pre-culture solution contained (g): glucose (10), yeast extract (2.5), K_2HPO_4 (5), KH_2PO_4 (3), NaCl (1), citrus pectin (7.5) and 10 ml trace mineral solution. 100 ml trace mineral solution contained (g):

$(NH_4)_2SO_4$ (0.2), KCl (0.5), $CaCl_2$ (0.1), $MgSO_4$ $7H_20$ (0.5), $ZnSO_4$ $7H_20$ (0.01) and $CuSO_4$ $7H_20$ (0.005). 3 L culture solution contained (g): yeast extract (7.5), K_2HPO_4 (15), KH_2PO_4 (9), NaCl (3) and 10 ml trace mineral solution.

At first four Erlenmeyer flasks (each containing 80 ml pre-culture, two with citrus pectin and two without citrus pectin) were inoculated with *A. foetidus.* The two flasks (one with pectin and other without pectin) were incubated in a rotary shaker at 150rpm and at 20^0C for 12 days. The remaining two flasks were incubated in a rotary shaker at 150rpm and at 25^0C for 12 days. Also, the *C. herbarum* was cultured in the same way.

After two days, each pre-culture was used to inoculate six Erlenmeyer flasks [two containing 1.5% Avicel (1.2g); two containing 1.5% corncob (1.2g); two containing 0.75% Pectin (0.6g) + 0.75% Sugar beet bulb (0.6g)]. One flask from each set was incubated in a rotary shaker at 150 rpm and at 20 and 25^0C. Samples for enzyme assay were taken after 5 and 12-days of incubation.

2.3.3. Xylanase activity

A 0.2 ml culture solution or enzyme was added to 1.8 ml substrate (1% xylan solution) and incubated at 40^0C for 5 min. At the end of 5 min, it was mixed with 3 ml DNS (dinitrosalicylic acid) reagent and boiled at 100^0C for 5 min. The absorbance was read at 540 nm and then translated (after subtracting the absorbance of reagent blank) into xylose (equivalent) using a standard graph.

2.3.4. β-glycosidase, β-xylosidase and α-arabinofuranosidase activities

For the determination of β-glycosidase activity, a 25 µl culture solution or enzyme was added with 25 µl of 200 mM sodium acetate buffer pH 5.0 and 50 µl water and pre-incubated at 37^0C for 10 min. This was followed by the addition of 25 µl p-nitrophenyl *β*-glycoside (pNpG) (in case of β-xylosidase activity: p-nitrophenyl *β*-D-xylopyranoside and in case of α-arabinofuranosidase activity: p-nitrophenyl α-arabinofuranoside) and incubated at 37^0C for 30 min. Finally, 100 µl of glycine-NaOH buffer pH 10.8 was added and the absorbance read at 405 nm. p-Nitrophenol was used as a standard.

2.3.5. Polygalacturonase activity

For the determination of polygalacturonase activity, following solutions were prepared: a) 75 mM sodium acetate buffer, containing 7.5 mM EDTA and 0.3% polygalacturonic acid (w/v) pH 5.1; b) 0.6 M NaCl solution. The solutions (a) and (b) were mixed at a ratio of 80:20 respectively and used as a substrate. A 0.1 ml culture solution or enzyme was added to 0.4 ml H_2O and 2.5 ml substrate and incubated at 40^0C for 30 min. The reaction mixture was boiled at 100^0C for 10 min. A 0.5 ml aliquot was taken and continued as described [22]. Finally, the reaction mixture was centrifuged at 3000 rpm. The absorbance was read at 520 nm and the values were translated (after subtracting the absorbance of reagent blank) into galacturonic acid (equivalent) using a standard graph.

2.3.6. Preparation of culture filtrate

A. foetidus and *C. herbarum* cultures (after 5 days of growth) were filtered through glass wool. The culture filtrates obtained (3120 and 3000 ml respectively) were further filtered through a millipore filter (size 0.2 μM) and concentrated to 870 ml each by ultra-filtration.

2.3.7. Ammonium sulphate precipitation and desalting

The proteins present in the concentrated culture filtrates were precipitated at 4^0C over night by adding 449.74g of $(NH_4)_2SO_4$ (80% saturation). Obtained precipitates were dissolved in water and desalted using Bio-Gel P-6 (2.6x100sm) column at a flow rate of 30 ml/h. The active fractions were collected and lyophilized.

2.3.8. Determination of pH and temperature optima

The optimum temperature was determined by carrying out the standard assays at various temperatures between 10 and 60^0C in 0.2 M Na acetate buffer, pH 5.0. The pH optimum was determined by measuring activity at 40^0C using 0.05 M sodium acetate (pH 3.0-9.0) and 0.05 M sodium phosphate buffer (pH 3.0-6.0 and 6.0-7.0).

2.4. Isolation of lignin-peroxidase from fungus *Pleurotus ostreatus*

2.4.1. Chemicals

Sorbents Sephadex G-10, TSK HW-55f and DEAE-TSK HW-650S were from “Pharmacia” Sweden and “Toyo Soda Company” Japan. SDS-PG was prepared as described [21]. Fungus *Pleurotus ostreatus* was from the Institute of Microbiology Academy of Sciences Republic of Uzbekistan. All the remaining chemicals were AnalaR grade and from Sigma Chemical Company.

2.4.2. Growth conditions

Pleurotus ostreatus was maintained in a splay wort-agar gel. Cultivation was carried out using 750 ml Erlenmeyer flask at 240 rpm and 30^0C for 17 days. The growth medium contained the agriculture byproducts such as pressed cake from seeds of cotton plant, birch rasps in different concentration, with a different degree of dispersion of particles as carbon source. Culture samples were taken for analyses at 24 h intervals.

2.4.3. Lignin-peroxidase activity

Lignin-peroxidase assay was carried out as described [24].

2.4.4. Ammonium sulphate precipitation and desalting

Precipitation was carried out at 20, 40 and 60% $(NH_4)_2SO_4$ saturation. Active fraction was obtained at 40% $(NH_4)_2SO_4$ and this was dissolved in

water (10 ml) and desalted on a Sephadex G-10 column (2.6x50 cm), at a flow rate of 30 ml/h. Protein was detected at 280 nm with a sensitivity of 0.1. Active fractions were pooled and freeze dried.

2.4.5. Gel filtration

A 10.72 mg freeze-dried sample was dissolved in 5 ml 0.01M Na acetate buffer, pH 4.9 and applied on to a TSK HW-55f column (1.6x125 cm), equilibrated with the same buffer and eluted at a flow rate of 30 ml/h. The protein was detected at 280 nm with a sensitivity of 0.1.

2.4.6. Ion exchange chromatography

A 5.69 mg active fraction from the above step was applied on to a DEAE-TSK HW-650S column (1.6x15 cm), equilibrated with 0.01 M Na acetate buffer, pH 4.9. The column was washed with the above buffer and eluted with 500 ml 0-0.5 M NaCl prepared in the same buffer, at a flow rate of 60 ml/h. The protein was detected at 280 nm with a sensitivity of 0.1.

2.4.7. Determination of pH and temperature optima

The optimum temperature for activity was determined by carrying out the standard assays at various temperatures between 20 and 50^0C in 0.05 M Na acetate buffer, pH 4.5. The optimum pH was determined by measuring the activity at 40^0C between the pH 2.0 and 5.0 using 0.05 M Na acetate and 0.05 M sodium phosphate buffers between the pH 6.0 and 7.0.

2.5. Enzyme treatment of tomato and pomegranate seeds

2.5.1. Enzyme treatment of isolated ligno-carbohydrate part from tomato and pomegranate seed shells

Enzyme treatment was carried out using cell wall degrading enzymes from *A. terreus, A. foetidus, C. herbarum* and *P. ostreatus*. For this purpose, enzymes were produced in a large scale using ligno-carbohydrate part from tomato seed shells as a carbon source. Enzymes obtained from *A. terreus, A. foetidus, C. herbarum* and *P. ostreatus* were taken 3; 6; 3; and 9 mg at a ratio of 1:2:1:3, respectively and dissolved in 2 ml 30 mM Na acetate buffer, pH 5. This was added to 840 mg substrate and incubated at 30^0C for 72 hrs. The enzyme substrate ratio was 1:40. Aliquots were taken after 24, 48 and 72 hrs and the sugars released determined.

2.5.2. Preparation of material

The fresh seeds of tomato and pomegranate (20 kg each) were carefully washed with water to remove any surface contaminations and dried at 22^0C for 30 days.

2.5.3. Enzyme treatment of tomato and pomegranate seed shells

A 1 kg dried tomato or pomegranate seed was placed in a 5 L glass container and added with water up to 2.5 L. To this a 2.5 mg enzyme sample was added and incubated at 40^0C for 48 hrs. The enzyme sample used was from *A. terreus, A. foetidus, C. herbarum* and *P. ostreatus* at a ratio of 1:2:1:3. The procedure was repeated 4 times to each seed. The respective seeds without the enzyme were used as blanks. At the end of incubation, seeds were filtered and washed with water and dried at 22^0C for 30 days.

2.5.4. Weight loss of seeds

This was determined by gravimetric method.

2.5.5. Examination by electron-microscope

Tomato and pomegranate seeds, before and after the enzyme treatment were examined at low vacuum and at accelerating voltage of 20 kV by electron-microscope LEO 900 (Zeiss).

2.6. Oil production by cold pressing

For this purpose, the equipment designed and constructed at the Institute of Electronics (Uzbekistan, Tashkent) was used. The screw press-granulator is the main feature of this equipment, which consists of frame, reducer, screw roller, pressing cage, and oil cake forming matrix. The productivity of the expeller is 100 kg/h.

During the design of this equipment, new technical decisions were made. These included the design of pressing cage and auger roller, which drastically reduced the fabrication expenses and optimized the operating parameters [25]. Device for seed oil production by cold pressing consists of press-granulator, which works on a scheme of frequent pressing. During pressing, calibrated granules are produced by giving the form and its size, which would depend on the hole in matrix-draw plate.

Press-granulator (Fig.1) consists of a frame (1), shortcut auger roller (2), triple-transit whorl (3), and a matrix (4). The matrix is made in the flange manner and is directly bolted on to frame by spire. Moreover, between triple-transit whorl and matrix (7), clearance (5) installed with no more than 0.5 mm, an end bearing in the bronze bushing manner, installed to matrix. The pressing cage (6) is made of two identical semi cylinder and having vertical connector. On the plane of the connector of semi cylinder is placed the steel figured knives, which have emerging to pressing cage part and being situated in gap between auger whorl and intended for stopping of rotary action of pressing material. A ring with eight hole (9), on which installed knives for granule cutting (8) is present. Moreover, the ring does the role of buffer puck between external stubborn bearing and mounting nut on roller and serves the lid on bearing jack.

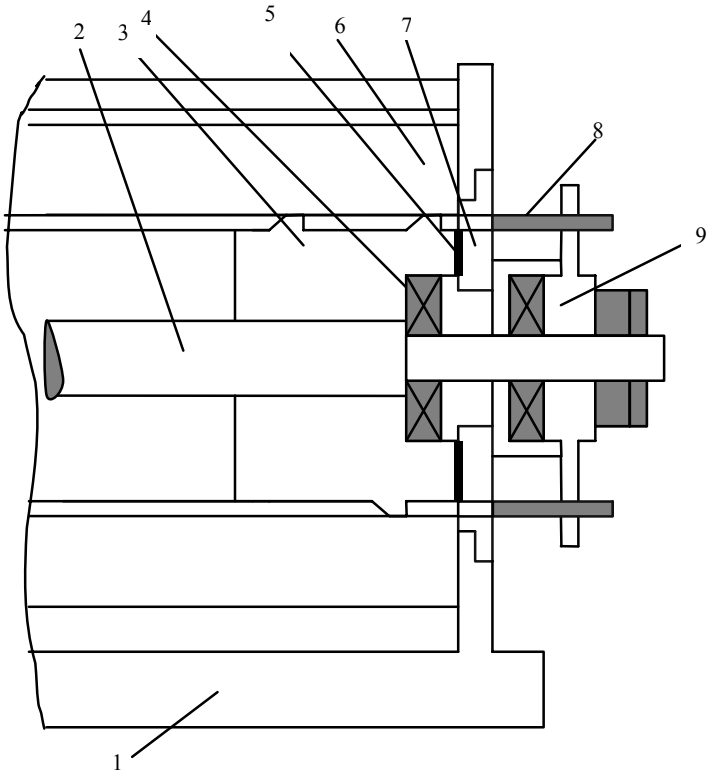

Figure 1. Oil pressing mechanism of the press-granulator for cold pressing.

Press-granulator works by frequent pressing of the material without preliminary heating. Triple-transit whorl of the pressing cage creates pressure at the end of press tract, which is directed to the matrix and promotes the withdrawal of processed material from pressing zone. Such condition facilitates to obtain strictly calibrated granules depending on the form and size of the hole in matrix, as well as making uniform oil cake structure. This improved the technology for seed processing by scheme of frequent pressing.

The press-granulator has the following technical characteristics: The length of pressing cage - 560mm; Press section number - 2; Numbers of screw roller - 6; Screw roller's rotation frequency - 28 rpm; Number of holes in matrix - 36; Number of knifes for granule cutting - 4 and length of granules - 17-23mm.

2.7. Determination of nutritional qualities of oil

2.7.1. Determination of tomato and pomegranate seed oil fatty-acid composition

0.2 ml tomato or pomegranate oil was dissolved in 2 ml diethyl ether and added with 0.1 ml 10% KOH in methanol and boiled at 100^0C for 3 min. A 0.2 ml hexane and 1 ml water were added. The hexane phase was separated and 2

μl of which was analysed by Mass spectrometry - GC-MS "Hewlett Packard" HP 5972 with HP–5 MS (30m x 0.25 mm) column and motionless liquid phase (5% phenyl methyl silicone). Temperatures: in evaporator - 280^0C; in thermostat: 2 min 100^0C - 250^0C; 5 min 250^0C; in interface - 300^0C.

2.7.2. Microelement composition analysis

0.1 ml tomato or pomegranate oil was processed by HNO_3 and dissolved in de-ionized water and made up to 100 ml. Samples were analysed by ICP–MS AT 7500 "Agilent Technologies" (mass-spectrometry of inductive combined plasma). Plasma parameters: Power - 1200 volt; plasma - argon 14 L/min; gas - argon 1.2 L/min. Samples applied through peristaltic pump at a flow rate 0.1 prs. Management system for data processing: ICP - MS ChemStation A. 07. 01.

2.7.3. Vitamin composition analysis

Vitamin content in samples was determined by HPLC (Du Pont) method. Eluent: 0.25M sodium phosphate buffer, pH 3.5. Gradient: 70% methanol. Column: Hypersil ODS - C_{18} (12cm x 2mm). Column temperature: 30^0C. Samples for analysis were of 5 μl each.

3. Results and discussion

3.1. Isolation and characterization of ligno-carbohydrate part from tomato and pomegranate seed shells

It is well known that plant cell wall consists of polysaccharides, lignin and tannin. Cellulose is the main component of polysaccharide complex and corresponds up to 90% of the seed cell wall. Besides cellulose, the polysaccharide complex includes hemicellulose and pectin [25-29]. The second main component of seed cell wall is lignin, while tannin is a minor component. The covalent cross-link between lignin and polysaccharides provide hardness in seed shells, and hinder the degradation processes [30-32]. For effective destruction of seed cell wall by enzymes, it is necessary to determine the quantitative ratio of cell wall components, polysaccharides and lignin. The objective of this study was the enzymatic hydrolysis of tomato and pomegranate seed shells since these crops are widespread in Uzbekistan and their oils possess high biological value. For this purpose, it was necessary to investigate the chemical composition of tomato and pomegranate seed shells.

The dried tomato and pomegranate seeds after grinding and sequentially de-oiling by hexane and chloroform, were extracted using ethanol and benzene mixture (1:2) for 24 h, followed by drying and crushing in a grinding mill to powder. The output of crushed tomato and pomegranate seeds were 87 and 82% of the initial seed weight, respectively. For obtaining the sulphuric-acidic

Table 1. Chemical composition of tomato and pomegranate seeds (%).

Seeds	Komarov lignin	Wilshtetter lignin	Cellulose	RHP	NRHP
Tomato	11.51	5.2	15.33	7.87	20.04
Pomegranate	21.44	5.4	18.71	13.05	31.36

lignin (Komarov lignin), 1 g seed powder was processed with 13.2 M H_2SO_4 for 2.5 h at 25^0C as described [16] and the lignin obtained was neutralized with hot water and dried. The Komarov lignin in tomato and pomegranate seeds was 11.5 and 21.4%, respectively (Table 1).

Also, a detailed analysis of hydrochloro-acidic lignin (Wilshtetter lignin) was carried out. For this purpose, 50 g of tomato or pomegranate seed powder was treated with 480 ml HCl for 35 min [17] and after filtration the residue was suspended with 1310 ml 5% H_2SO_4. The suspension was boiled for 5-6 hour and the product was neutralized and dried. The Wilshtetter lignin isolated from tomato and pomegranate seed powders had dark pink and brown colours, respectively. Both lignins were diluted in dioxane - water mix (9:1), DMSO and alkali solutions. The yield of hydrochloro-acidic lignin from tomato and pomegranate seeds were 5.2 and 5.4% compared to 11.5 and 21.4% of Komarov lignin.

The molecular-mass distribution for isolated hydrochloro-acidic lignin was determined. For this purpose, 0.5% solution of hydrochloro-acidic lignin in DMSO was prepared and applied on to Sephadex G-75 (1.6x100cm) and the molecular mass determined as described [18]. The average number (Mn) and molecular mass (Mw) of hydrochloro-acidic tomato lignin were 2700 and 4100, while Mn and Mw for the pomegranate lignin were 5800 and 11000 respectively. The degree of polydispersity of the indicated lignin (Mw/Mn) was 1.5 and 1.9, respectively.

Following de-lignification, the isolation of polysaccharide from tomato and pomegranate seed powders was performed. For this purpose, 1 g seed powder was treated with 25 ml mixture of HNO_3 and ethanol (1:4) for 1 hour as described [16]. The completion of de-lignification was determined by the absence of red colour with the addition of phloroglucinol [16]. The cellulose content of tomato and pomegranate seeds was 15.3 and 18.7%, respectively.

For further characterization, the polysaccharide complex was separated in to RHP and NRHP fractions. A 5 g polysaccharide complex was treated with 200 ml of 2% HCl for 3 hrs. The mixture was filtered, neutralized by 10 ml 20% NaOH and analyzed. In the second step, the residue from above step was hydrolyzed first with 40 ml 80% H_2SO_4 at room temperature for 3 hr and after the addition of 600 ml water for 5 hr at 100^0C. The reaction mixture was filtered, and the filtrate neutralized by 10 ml 20% NaOH and analyzed. The RHP content in tomato and pomegranate seed polysaccharides were 7.9 and 13.1%, while the NRHP content was 20.1 and 31.4%, respectively.

Table 2. Functional composition of the tomato and pomegranate seed lignin (%).

Seeds	OH	CO	COOH
Tomato	8.17	2.61	0.13
Pomegranate	10.24	3.52	0.18

Further study included the analysis of OH, CO and COOH groups in hydrochloro-acidic lignins by acetylation, oxidization and chemo sorption methods and the results presented in Table 2.

As shown in Table 2, Wilshtetter lignins contain main functional groups. The presence of OH groups in both cases was approximately 3 times higher than CO groups and the availability of COOH groups distinguishes them from lignin from cotton and other plants [33].

The Infra Red spectra analysis revealed that (a) tomato seed Wilshtetter lignin has (cm^{-1}): 3343, 3306ν (characteristic for OH groups in phenol compounds); 2941, 2882ν (C–H bonds); 1651ν (CO groups); 1542ν (skeletal vibration of the aromatic ring); 1429, 1370, 1319ν (δ C–H bonds); 1263, 1162, 1111ν (C–O–C, C–C and C–O bonds).

b) Pomegranate seed Wilshtetter lignin has (cm^{-1}): 3354ν (characteristic for OH groups in phenol compounds); 2940, 2894ν (C–H bonds); 1736, 1656, 1639ν (CO groups); 1510 (skeletal vibration of the aromatic ring); 1464, 1426, 1371, 1334 (δ C–H bonds); 1266, 1232, 1161, 1113ν (C–O–C, C–C and C–O bonds). It is necessary to point out that the frequencies in the area of 1500–1610 cm^{-1} are the characteristic of lignins.

For detailed analysis, lignins were subjected to alkaline hydrolysis by 8% NaOH at 100 ^{0}C for 1 hr as described in methods section. The alkaline treatment under these conditions results in the hydrolysis of ester bonds between lignin and phenolic acids. For example, in wheat straw, p-coumaric acid bound to lignin was identified [34]. It is known that p-coumaric acid linked to lignin [35-37], whereas the ferulic acid generally linked to hemicelluloses [38], which can be identified in many non-lignin tissues [39]. The sum of the isolated phenoloxy-acids and suspension particles are shown in Table 3.

Suspension particles were derived by acidifying with H_2SO_4 after alkaline hydrolysis. These represent dark brown amorphous powders, which were insoluble in water and organic solvents and poorly soluble in alkali solutions. Probably,

Table 3. Products of alkaline hydrolysis of tomato and pomegranate seeds in grams.

Seeds	Fixed residue	Suspension particles	Sum of phenol hydroxy-acids
Tomato	1.3072	0.3196	0.0432
Pomegranate	2.7595	1.1612	0.0224

suspension particles could be derived as a result of lignin - carbohydrate complex hydrolysis and consequently were their small pieces. Seed lignin components could be identified as small and large particles of high polymer or colloid.

The presence of lignin in suspension particles was established by phloroglucinol reaction (red colour) [16]. Therefore, tomato and pomegranate seed suspension particles isolated during alkaline hydrolysis were small fragments of their lignin - carbohydrate complex. The colloidal state of these fragments was stabilized by a protective action of polysaccharides [40]. By acidifying, as mentioned above or at cryogenic processing, the colloidal state of suspension particles changes [20] their settling. Therefore, the purpose of analysis of the chemical nature of suspension particles was to treat by 2 N H_2SO_4 at 100^0C for 48 h. After the hydrolysis by phenol sulfuric method, the carbohydrates were analyzed by paper chromatography, which included glucose, xylose, arabinose and galactose.

The IR spectra analysis of suspension particles from tomato and pomegranate seeds suggests the characteristic vibration for lignins and carbohydrates:

a) Tomato lignin suspension particles have (sm^{-1}): 3752, 3737, 3713, 3650, 3287ν (characteristic for OH groups in phenol compounds); 2926, 2854ν (C-H bonds); 1716, 1654ν (CO and COOH groups); 1543, 1517ν (skeletal vibration of the aromatic ring); 1458ν (δ C-H bonds); 1147ν (C-O-C, C-C and C-O bonds) and 1051ν (skeletal vibration of the pyranose ring).
b) Pomegranate lignin suspension particles have (sm^{-1}): 3421, 3752, 3737, 3713, 3650 (characteristic for OH groups in phenol compounds); 2854, 2922 (C-H bonds); 1717, 1654 (CO and COOH groups); 1542, 1508 (skeletal vibration of the aromatic ring); 1458 (δ C-H bonds); 1122 (C-O-C, C-C and C-O bonds) and 1044 (skeletal vibration of the pyranose ring).

In contrast to IK spectra of lignins, IK spectra of the suspension particles have a more crumbling type in consequence of imposition signals of lignin and carbohydrate compounds of investigated samples and reveal the widening of absorption of the OH groups in this area of from 3200-3600 up to 3100-3700 sm^{-1}. Also, the molecular masses of the suspension particles were determined by viscosity method and were equal to 83,400 and 79,800 for tomato and pomegranate seeds, respectively.

3.2. Isolation of plant cell wall degrading enzymes from *Aspergillus terreus, Aspergillus foetidus* and *Cladosporium herbarum*

Based on our preliminary results, *Aspergillus terreus*, *Aspergillus foetidus* and *Cladosporium herbarum* were capable of producing high-level of

polysaccharidases. Also, different strains of these fungi and their enzyme systems have been isolated, characterized and used in many industrial applications [41-54]. Therefore, these fungi were selected for producing cell wall degrading enzymes.

3.2.1. Isolation of highly effective cellulase enzymes from fungus *Aspergillus terreus*

Aspergillus terreus has been reported to produce an effective cellulolytic enzyme system [55-61]. Depending on the cultivation conditions, it produces highly effective cellulase and xylanase for lignocellulose waste treatment [61] and plant cell wall degradation [60]. In the present study, the production of cellulolytic enzymes by *Aspergillus terreus* was monitored at different time intervals (7, 10, 14 and 17 days). The endoglucanase activity reached a maximum by day 10. Thus, for the isolation of cellulolytic enzymes, the fungus was grown for 10 days.

A 220 ml culture filtrate containing 106.1 units of total cellulase activity (table 4) and the specific activity of 0.688 units/mg protein was concentrated to 17 ml by rotary evaporation at 38^0C. Analysis of the concentrated sample by SDS-PAGE showed that it contained 9 proteins with molecular mass of 120, 80, 69, 45, 35, 30, 27, 20 and 17 kDas. Two active fractions were isolated as summarized in Table 4.

SDS-PAGE analysis showed that the active fraction I contained four proteins with molecular masses of 17, 30, 35 and 37 kDa, while fraction II was homogeneous with one protein band having a molecular mass of 30 kDa.

The pH and temperature optima of cellulase-I and II were determined. Both were optimally active at pH 4.3. The cellulase-I was more active than cellulase-II, but the latter enzyme showed broader pH optima (3.5-8.0) than former (3.5-6.0). Both enzymes were optimally active at 43ºC but the activity of cellulase-I decreased rapidly above 60ºC. The cellulose-I and II were obtained in large quantities for tomato and pomegranate seed shell degradation.

Table 4. Summary of purification of cellulase components from *A. terreus.*

Steps	Total activity	Total protein	Specific activity	Purification step	Recovery
	U/ml	mg	U/mg protein	Time	%
Culture solution	106.1/220	154	0.688	-	100
Desalting	66.47/150	63.38	1.048	1.52	62.64
Gel filtration	59.82/340	35.12	1.70	2.47	56.38
DEAE TSK HW 650 (Ion Exchange chromatography)					
Active fraction-1	3.01/10	3.41	0.88	1.28	2.83
Active fraction-2	24.03/80	0.96	25.03	36.38	22.65

3.2.2. Isolation of polysaccharidases from psychrophilic fungi *Aspergillus foetidus* and *Cladosporium herbarum*

The psychrophilic fungi *Aspergillus foetidus* and *Cladosporium herbarum* were grown as described in methods to monitor the production of polysaccharidases. 10 ml culture aliquots were withdrawn after 5 days of growth at different temperatures with different carbon sources and assayed for endoglucanase, xylanase, β-glucosidase, β-xylanase, α-arabinofuranosidase and polygalacturonase activities. The results are summarized in Table 5.

A. foetidus produced the highest level of endoglucanase, xylanase and β-xylosidase in 1.5% Corncob at 25^{0}C, while *C. herbarum* grown in medium containing 0.75% Pectin + 0.75% Sugar beet pulp at 20^{0}C and 25^{0}C produced

Table 5. Levels of polysaccharidases in the culture filtrates of *A. foetidus* and *C. herbarum.*

Enzyme	*A. foetidus*						*C. herbarum*					
	1.5% Avicel		1.5% Corncob		0.75% Pectin, 0.75% Sugar beet pulp		1.5% Avicel		1.5% Corncob		0.75% Pectin, 0.75% Sugar beet pulp	
	20^{0}C	25^{0}C	20^{0}C	**25^{0}C**	20^{0}C	25^{0}C	20^{0}C	25^{0}C	20^{0}C	25^{0}C	**20^{0}C**	25^{0}C
Endoglucanase	0.0246	0.04	0.288	**0.37**	0.1923	0.255	0.002	0	0.023	0	**0.19**	0
β-glycosidase	0.014	0.016	0.08	**0.092**	0.039	0.06	0.001	0.005	0.025	0.023	**0.470**	0.065
Xylanase	1.893	3.424	22.31	**23.49**	8.4606	13.952	0	0.079	0.256	0.1149	**0.13**	0.1478
β-xylosidase	0.0013	0.001	0.119	**0.14**	0.0063	0.01	0	0	0.0025	0.001	**0.012**	0.0012
α-arabino-furanosidase	0.0002	0.0001	0.054	**0.066**	0.1354	0.3046	0.0002	0	0.006	0	**0.11**	0.0057
Polygalac-turonase	0	0	0.051	0	0.4039	0.4835	0	0	0.1079	0.1201	**2.1384**	1.7340

Enzyme activity is given in unit/ml. Bold type indicates highly active enzymes

Table 6. Time course of accumulation of polysaccharidases in culture solutions of *A. foetidus* and *C. herbarum* after 12 days of growth.

Type of Activities	*A. foetidus*						*C. herbarum*					
	1.5% Avicel		1.5% Corncob		0.75% Pectin, 0.75% Sugar beet bulb		1.5% Avicel		1.5% Corncob		0.75% Pectin, 0.75% Sugar beet bulb	
	20^{0}C	25^{0}C	20^{0}C	**25^{0}C**	20^{0}C	25^{0}C	20^{0}C	25^{0}C	20^{0}C	25^{0}C	**20^{0}C**	25^{0}C
Endoglucanase	0.0324	0.0757	0.46	**0.4643**	0.330	0.274	0	0	0.0301	0.023	**0.0204**	0.0354
β-glycosidase	0.048	0.038	0.09	**0.093**	0.403	0.520	0.001	0.003	0.019	0.04	**0.098**	0.031
Xylanase	3.092	5.019	5.603	**5.618**	5.602	5.531	0	0.009	0.6943	0.1892	**0.249**	0.1776
β-xylosidase	0.006	0.009	0.466	**0.521**	0.048	0.0594	0	0	0.006	0.0025	**0.0139**	0.0006
α-arabino-furanosidase	0.0006	0.001	0.068	**0.073**	0.501	0.503	0.0009	0.001	0.031	0.015	**0.523**	0.004
Polygalac-turonase	0	0	0.0308	0.0278	0.8064	0.6687	0	0.036	0.0488	0.0773	**0.9062**	0.6114

Enzyme activity is given in unit/ml. Bold type indicates highly active enzymes.

the highest level of endoglucanase, β-glycosidase, xylanase, α-arabinofuranosidase and polygalacturonase.

After 12 days of growth, 10 ml aliquot was withdrawn and different activities were measured. The results are summarized in Table 6. The data show that some activities decreased after 12 days of growth.

For the isolation of polysaccharidases from *A. foetidus* and *C. herbarum,* the culture solutions obtained on 1.5% Corncob at 25^0C and 0.75% Pectin + 0.75% Sugar beet pulp at 20^0C after 5 day of growth were used. These were filtered through glass wool and the endoglucanase, xylanase, β-glycosidase, β-xylosidase, α-arabinofuranosidase and polygalacturonase activities were measured (Table 7). Also, the culture solutions were concentrated and the enzymes were partially purified (Table 7).

The temperature and pH optima of different activities were determined by measuring the activity between 10 and 70^0C and at pH between 2.5 and 7.0. The results are summarized in table 8.

Xylanase and polygalacturonase from *A. foetidus* showed maximum activity at 40^0C, while all other enzymes showed optimal activity at 60^0C. Also, these enzymes were maximally active at pH ranging between 2.6 and 4.8, except polygalacturonase, which was optimally active at pH 6.8. Xylanase and polygalacturonase from *C. herbarum* were optimally active at 50 and 40^0C as well as at pH 6.6 and 6.2, respectively.

Table 7. Purification of polysaccharidases from *A. foetidus* and *C. herbarum.*

Type of Activities	*Aspergillus foetidus* (1.5% corncob 25^0C)					
	Steps of purification					Recovery, %
	1 (3120 ml)	2 (2240 ml)	3 (870 ml)	4 (1050 ml)	**5 (75 ml)**	
Endoglucanase	2174.64	-	1549.47	-	**1355.36**	62.3
β-glycosidase	630.56	-	598.95	-	**533.32**	84.5
Xylanase	315756.48	1715.84	94248.84	-	**5330.85**	1.7
β-xylosidase	1257.84	-	1084.89	611.10	**397.71**	31.6
α-arabinofuranosidase	346.64	-	200.97	-	**155.24**	44.8
Polygalacturonase	197.18	-	85.93	-	**57.33**	29.1
Type of Activities	*Cladosporium herbarum* (0.75% Pectin+0.75% Sugar beet pulp 20^0C)					
	Steps of purification					Recovery, %
	1 (3000 ml)	2 (2120 ml)	3 (870 ml)	4 (1150 ml)	**5 (40 ml)**	
Endoglucanase	68.93	-	-	-	**-**	-
β-glycosidase	115.51	-	89.76	43.70	**36.36**	31.5
Xylanase	4236.10	1141.44	1445.84	-	**668.4**	15.8
β-xylosidase	-	-	-	-	**-**	-
α-arabinofuranosidase	-	-	-	-	**-**	-
Polygalacturonase	3606.00	-	2778.44	1024.42	**1442.24**	40

(1) Culture filtrate; (2) Ultra-filtrate; (3) Concentrate after ultra-filtration; (4) Supernatant after precipitation; (5) precipitate. Enzyme activities given as Units/total volume and bold type indicated final results.

Table 8. Temperature and pH optima of polysaccharidases.

Type of Activities	*Aspergillus foetidus*		*Cladosporium herbarum*	
	t^0C	pH	t^0C	pH
Endoglucanase	**60**	**2.6**	-	-
β-glycosidase	**60**	**3.4**	50	5.2
Xylanase	**40**	**4.8**	**50**	**6.6**
β-xylosidase	**60**	**3.4**	-	-
α-arabinofuranosidase	60	3.8	-	-
Polygalacturonase	40	6.8	**40**	**6.2**

Bold type indicates highly active enzymes

Thus, *Aspergillus foetidus* enzyme sample contained highly active endoglucanase, β-glycosidase, xylanase, β-xylosidase, α-arabinofuranosidase and polygalacturonase, while *Cladosporium herbarum* enzyme sample contained highly active xylanase and polygalacturonase. These enzyme mixtures were obtained in large quantities and used for tomato and pomegranate seed shell treatment.

3.3. Isolation of ligno-peroxidase enzyme from fungus *Pleurotus ostreatus*

Basidiomycetes produce an active enzyme system consisting hydrolytic oxidation-reduction enzymes, hydrolases and ligninases capable of degrading wood. Among them, the *Pleurotus ostreatus* is well known. Also, the mycelium of this fungus contains useful proteins, amino acids and specific macro and microelements [62]. The fungus grows well and utilizes complex and insoluble agricultural lignocellulosic wastes [63]. It showed peroxidase producing ability in different agricultural plant wastes of Uzbekistan [64,65]. The ability of *P. ostreatus* to produce lignoperoxidase using birch sawdust was investigated.

The production of lignoperoxidase in culture solution of *P. ostreatus* was studied during 21 days of culturing by taking an aliquot each day. The lignoperoxidase activity was detected on day 3, which reached a maximum on 17-18th day of incubation. Hence, *P. ostreatus* was cultured for 18-days to isolate and purify the lignoperoxidase.

The culture was first filtered through glass wool to separate the fungal biomass and then through nitrocellulose filter (0.4 micron). The lignoperoxidase was purified from the culture filtrate by gel filtration and ion-exchange chromatography as summarized in Table 9.

The purified enzyme was subjected to 10% SDS-electrophoresis. The enzyme was homogeneous with a molecular mass of 44,000 Da (Fig. 2).

The lignoperoxidase was optimally active at 29^{0}C and at pH 2.7 (Fig. 3). The enzyme showed very low level of activity at temperatures above 40^{o}C and

Table 9. Summary of purification of lignoperoxidase enzyme from fungus *P. ostreatus.*

Steps	Total activity	Total protein	Specific activity	Purification step	Recovery
	U/ml	mg	U/mg protein	time	%
Culture solution	233.0/170	54.06	4.31	-	100
Desalting	102.8/36	10.72	9.59	2.23	44.1
Gel filtration	89.5/63	5.69	15.73	3.65	38.4
Ion Exchange chromatography	64.8/40	2.4	27.0	6.26	27.8

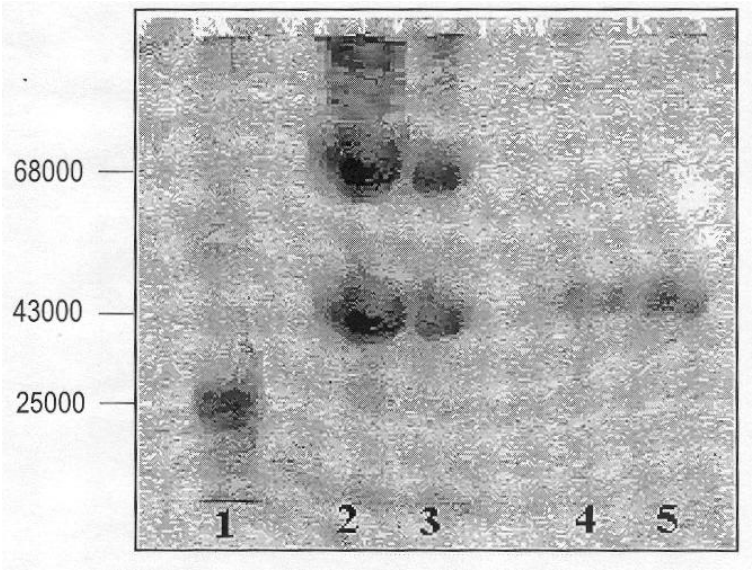

Figure 2. SDS – PAGE: Lanes 1, 2 (50 μg) and 3 (25 μg) are standard markers. Lanes 4 (10 μg) and 5 (20 μg) are active fractions after Ion Exchange chromatography.

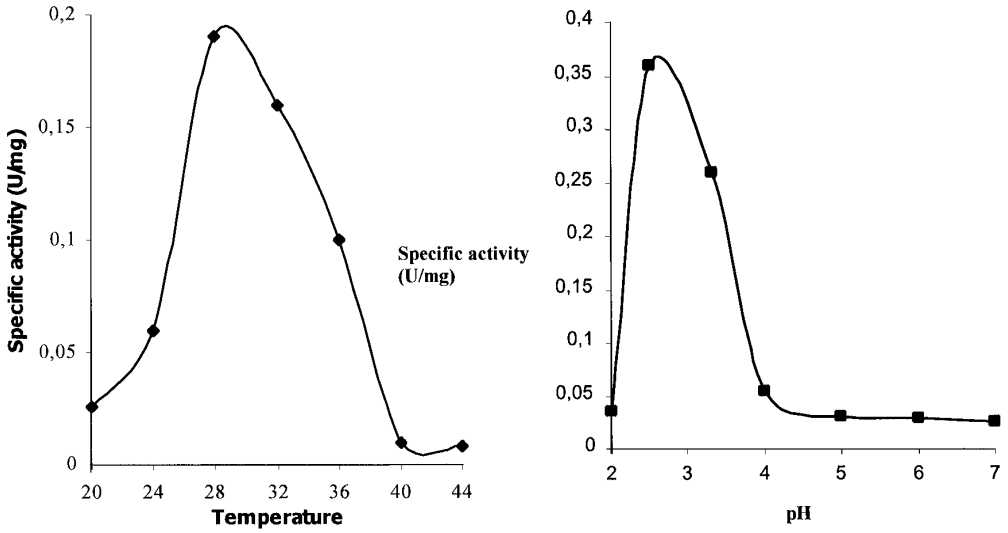

Figure 3. Effect of temperature and pH on the activity of lignoperoxidase.

pH >4.0. The enzyme from *P. ostreatus* was produced in bulk quantities and used for tomato and pomegranate seed cell wall degradation.

3.4. Enzyme solubilization of tomato and pomegranate seed shells

It is noteworthy that the cellulase, hemicellulase and pectinase obtained in the previous steps could be used in different industrial applications. Similar enzymes obtained from fungi and bacteria were successfully used in textile [66], paper [67], plant waste processing [68], food [69-76] and pharmaceutical [77-79] industries. Hence, the isolated polysaccharidases were tested for their efficiency to solubilize tomato and pomegranate seed shells with the aim of increasing the yield of oil during cold pressing.

Initially the influence of enzymes on isolated ligno-carbohydrate part of tomato and pomegranate seed shells was studied and the hydrolysis conditions were optimized. Subsequently, the enzyme treatment and solubilization of tomato and pomegranate seed shells were performed under optimal conditions.

3.4.1. Enzyme hydrolysis of isolated ligno-carbohydrate part of tomato and pomegranate seed shells

Enzyme treatment of ligno-carbohydrate part of tomato and pomegranate seed shells were carried out at pH 3.0, 4.0 and 5.0 and at 20, 30 and 40^{0}C for 72 hr as described in methods. The products released at different time intervals were determined and presented in Figs. 4 and 5.

Under all conditions, the selected combination of enzymes hydrolyzed ligno-carbohydrate part of tomato and pomegranate seeds (Figs. 4 and 5). The

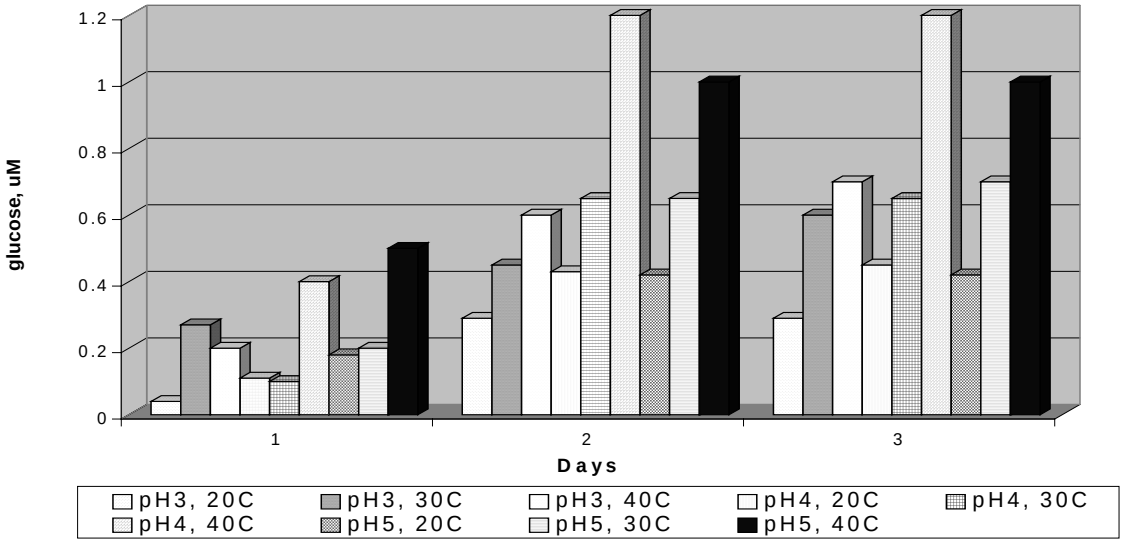

Figure 4. Enzymatic hydrolysis of ligno-carbohydrate part of tomato seed shells.

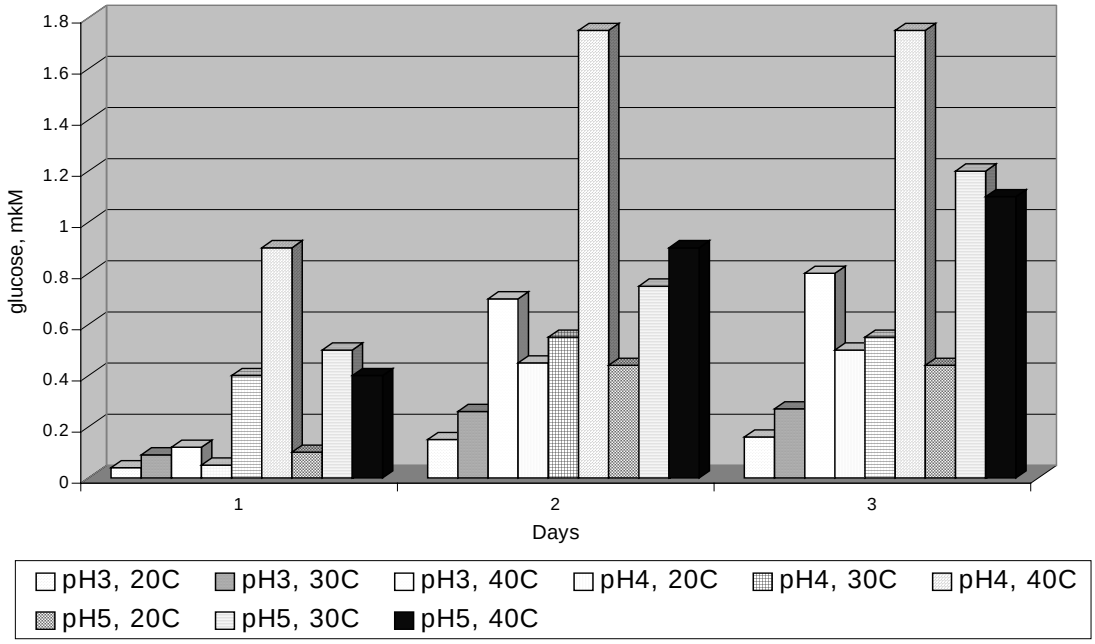

Figure 5. Enzyme hydrolysis of ligno-carbohydrate part from pomegranate seed shells.

highest amount of glucose from both substrates was released at pH 4.0 and 5.0 at 40^0C and after 2 days of incubation. Thus, pH 4.0 and 40^oC were selected as optimal conditions for the hydrolysis of both seed shells.

3.4.2. Enzymatic degradation of tomato and pomegranate seed shells

The fresh seeds (20 kg each) of tomato and pomegranate were thoroughly washed in water and dried at 22^0C for 30 days before subjecting to enzyme treatment at pH 4.0 and at 40^0C for 48 hr. The seeds were filtered and washed under running water and dried as above. The weight loss determined by gravimetric method showed 11.6 and 15.7% reduction in weight in case of tomato and pomegranate seeds, respectively.

The cell wall destruction of tomato and pomegranate seeds by enzyme treatment was examined under electron microscope. The control tomato seeds showed intact and closely packed fibers (Fig. 6, left), while no such intact fibers were seen with seeds treated by enzymes (Fig.7, left). Also, after enzyme treatment, the tomato seed shell surface appeared to be monotonous (Fig.8, left).

The difference between control pomegranate and tomato seeds was that the pomegranate seeds had highly packed surface with characteristic polyhedral parts compared to tomato seeds (Fig. 6). After the enzyme treatment, pomegranate seeds showed irregular nature with deepened parts of hydrolyzed surfaces between polyhedral parts (Fig. 7, right). After the enzyme

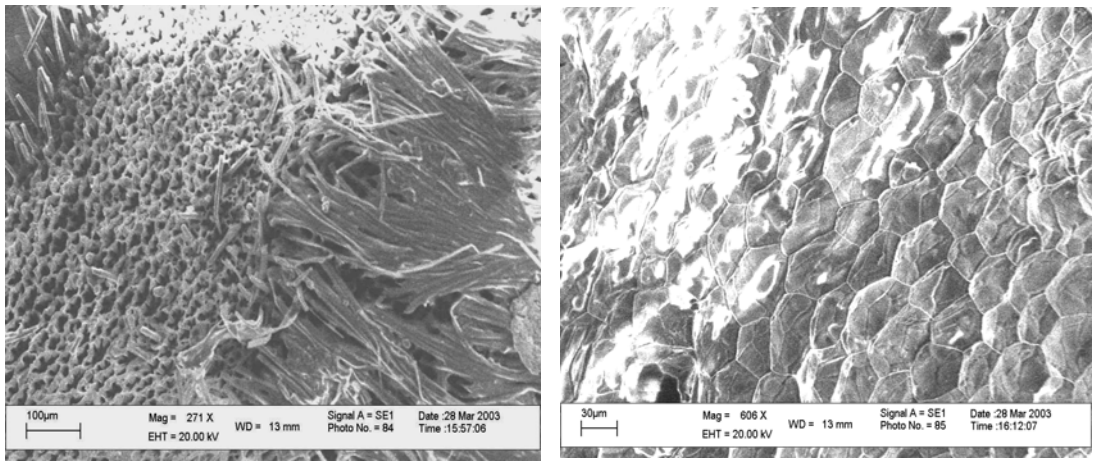

Figure 6. Tomato (left) and pomegranate (right) seed surfaces before enzyme treatment.

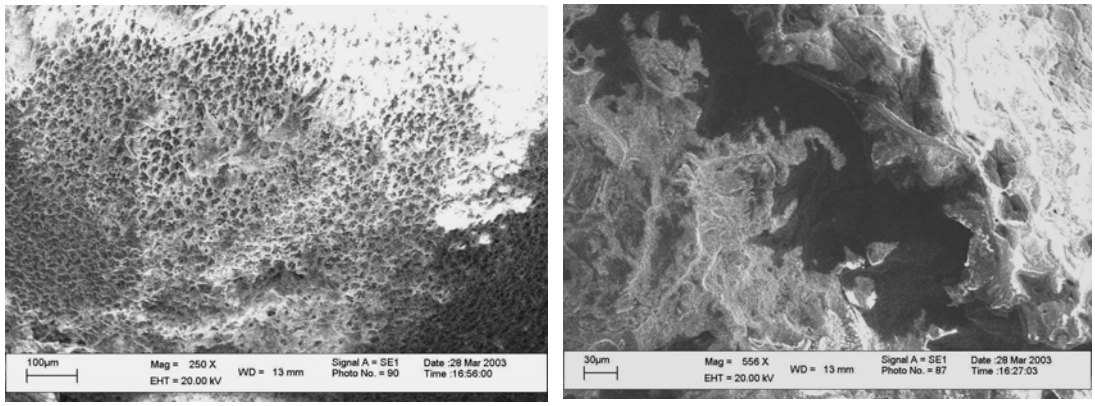

Figure 7. Tomato (left) and pomegranate (right) seed surfaces after enzyme treatment.

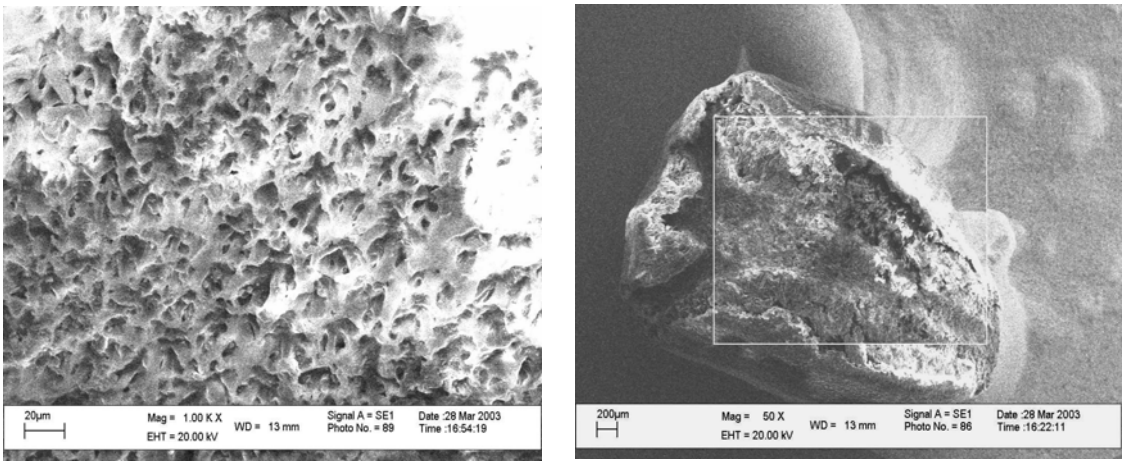

Figure 8. Damaged surface areas of tomato (left) and pomegranate (right) seeds after enzyme treatment in a large scale.

treatment, the observed difference between tomato and pomegranate seed shells could be due to the content and packing of polysaccharides and lignin in seed shells.

3.5. Evaluation of the role of plant cell wall degrading enzymes on yield and quality of oil from tomato and pomegranate seeds by cold pressing

3.5.1. Production of oil from enzyme treated tomato and pomegranate seeds

Cold pressing method for the extraction of plant oils is used mainly by small industries [87]. Two types of screw presses used [88] differ in the screw type and the oil outlet. The oil outlet is built like a strainer, where the press cylinder created by metal-bars placed close to each other. The gaps between the bars form the oil outlet, which can be varied according to the type of oil seed. The cake pressed out to an adjustable choke formed into plates (a kind of chips). The oil outlet from the other type of oil press consists of drilled holes in a special part of the cylinder tube. The pressed cake is forced out in the form of pellets through a changeable nozzle at the end of the cylinder. In order to avoid the blockage of the press cake outlet for some types of screw presses, it is necessary to heat this part of the press. The heating should be in the range between 60 and 80^0C. A higher temperature on the press cake outlet will lead to higher phosphor content in the oil. Also, this temperature has an effect on the oil temperature, which should not rise over 40^0C.

With a lower temperature on the cake outlet, the solid content in the oil increases. The nozzle diameter and gap-size of the choke depends on the seed used. The optimum nozzle diameter varies between 6 and 8 mm. With a nozzle diameter of 6 mm, temperature at 60^0C and a low humidity in the seed will minimize the phosphor content in the oil. Although, this may pose a high risk for nozzle blocking, the seeds are pressed harder by a smaller cake-outlet with high oil yield besides decreased capacity.

An important parameter to consider during the extraction of oil from seeds is the residual oil in the cake. A minimum of 10% oil in the cake is achievable by hard pressing with high percentage of oil yield. The hard pressing is achieved by changing the nozzle diameter. For an increased pressure, it is necessary to adjust the cake outlet by changing single segments of the screw in a strainer press. If the throughput is reduced (e.g. screw rotation speed is reduced), yield of oil is increased but the solid content in the oil is also increased. Alternatively, if the throughput is increased, the yield is reduced and the solid content in the oil is reduced. It is possible to find an optimal compromise according to individual aims with a revolution-regulated press-screw. This also allows pressing of a wide range of different oil seeds. Favorable

rotation speeds for the screw is between 20 and 50 rpm. In this range of rotation, a minimum energy is required. The higher the throughput of seed, the greater the capacity of the oil cleaning installation must be. With this increasing oil production, the total quantity of solids in the oil rises too [88].

In the present study, equipment designed and built at the Institute of Electronics (Uzbekistan, Tashkent) was used for oil production by cold pressing. The screw press-granulator is a main device in this equipment. This consists of a frame, reducer, screw roller, pressing cage, oil cake forming matrix and productivity of the expeller is 100 kg/h. The Design of muff of the pressing cage drum acts as a radiator in the system and during oil processing the temperature balance is maintained.

The optimum correlation between heat-generator and dissipation was determined during processing of sesame seed by selecting roller rotation speed and full size of the dens in matrix press. The parameters studied included (a) seed moisture and temperature before pressing, (b) oil and cake temperature after pressing, and (c) granulator matrix temperature. The data for sesame seed are presented in Fig. 9.

The pressing matrix and oil cake temperature curves are close to each other (Fig.9), which shows that an increase in oil cake temperature occurs during the formation of oil cake granules in matrix. This confirms the difference between the temperature of oil and oil cake.

The tomato and pomegranate seeds before and after their enzyme treatment were subjected to cold pressing. The optimum moisture for tomato and pomegranate seeds was 8.5 and 10%, respectively. Processing was carried out

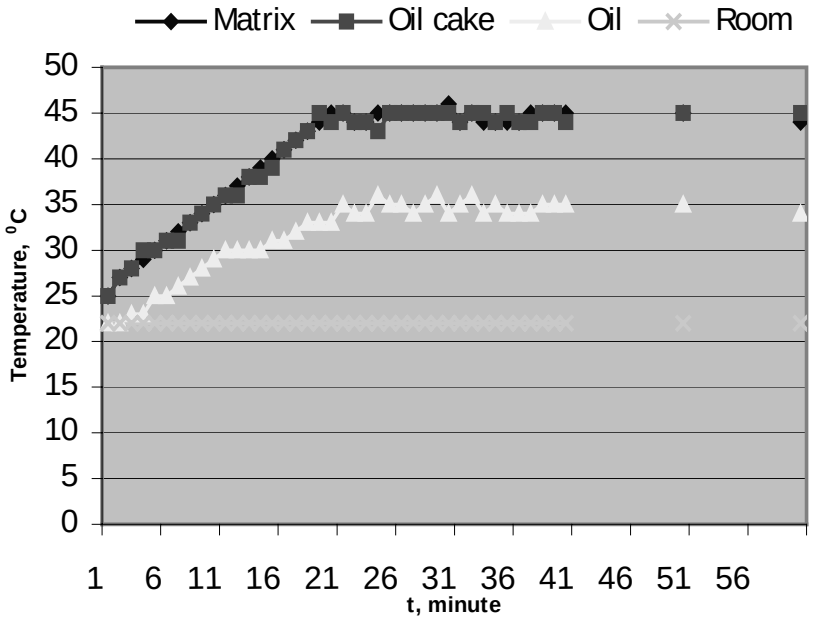

Figure 9. Dynamical change of temperature during cold pressing.

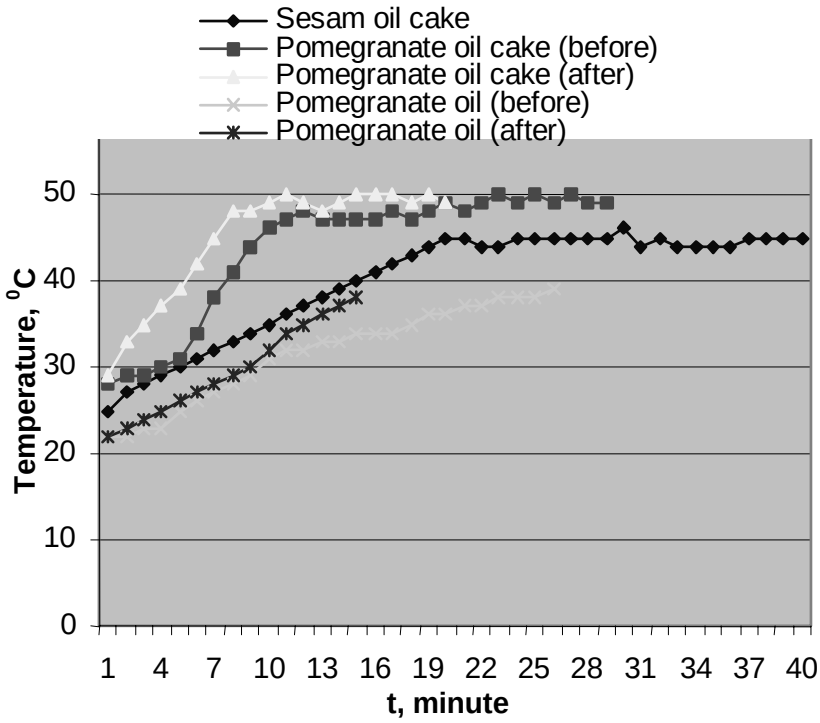

Figure 10. Dynamical change of temperature for oil cake and oil from pomegranate seeds during cold pressing.

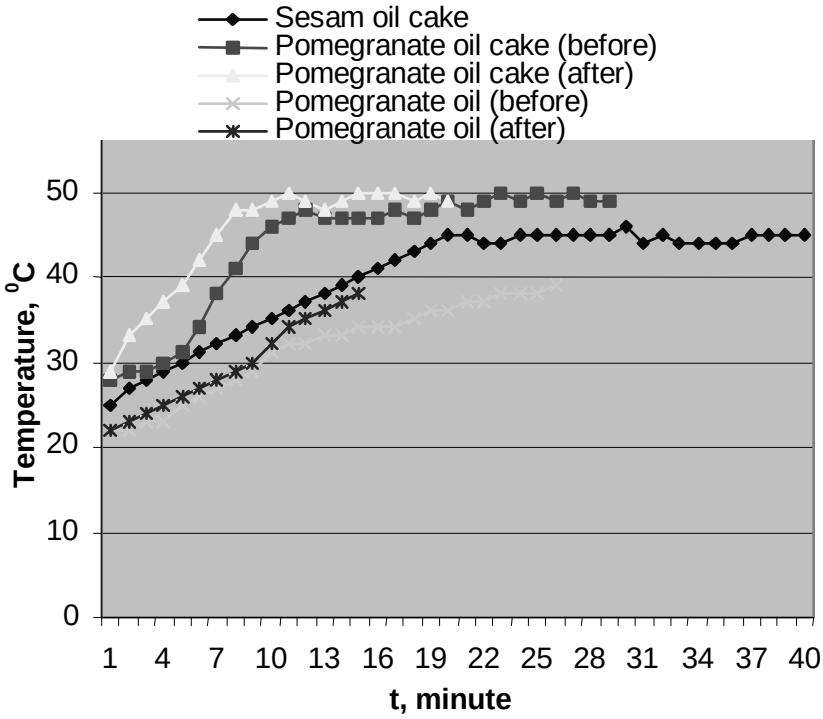

Figure 11. Dynamical change of temperature for oil cake and oil from tomato seeds during cold pressing.

according to scheme 1 and by five-fold re-pressing. This scheme guarantees the extraction of oil with a maximum efficiency. The temperature curves for cake and oil from tomato and pomegranate seeds were generated (Figs. 10 and 11).

Both tomato and pomegranate seeds treated with enzymes showed a large difference in temperature under pressing compared to control. This indicates that enzyme treatment destroyed the structural integrity of tomato and pomegranate seed shells. The data shows that after pressing 3 times, almost all the oil from enzyme treated pomegranate and tomato seeds was extracted (Figs. 12 and 13). While the oil extraction from non-treated seeds requires up to 5 re-pressing steps. Also, the data are in accordance with the changes in temperature curves.

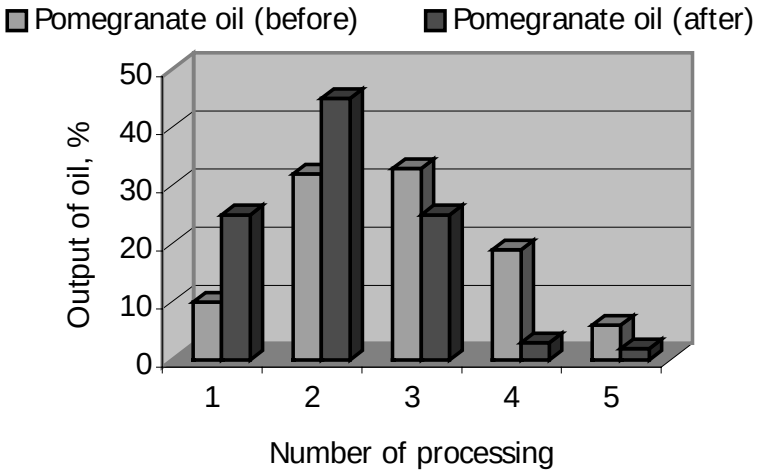

Figure 12. The yield of the pomegranate oil before and after enzyme treatment during cold pressing.

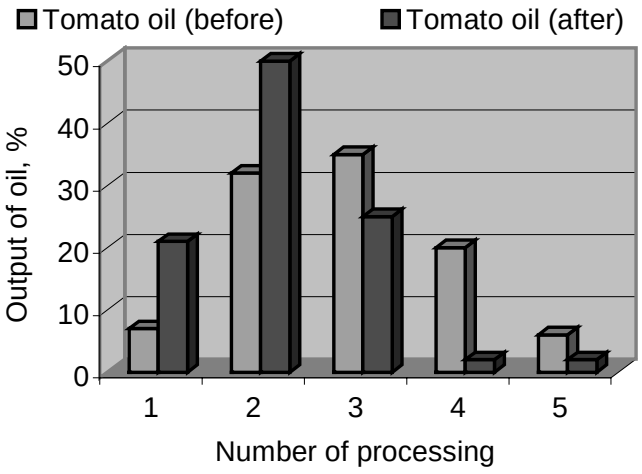

Figure 13. The yield of the tomato oil before and after enzyme treatment during cold pressing.

Table 10. Oil yield in tomato and pomegranate seeds.

Seeds	Initial oil content in seeds, %			The yield of oil after cold pressing, %		
	Before treatment	After treatment	The change of oil content	Before treatment	After treatment	The change of oil content
Tomato	20.5*	25.4*	23.9*	14.2	19.3	**34.5**
Pomegranate	15.6*	16.3*	4.5*	2.1	3.5	**66.7**

(*) It is determined by chemical extraction method;

The threshold oil content is the minimal oil content in cake after pressing. In case of tomato and pomegranate seeds, the threshold oil content before enzyme treatment was 6.3 and 13.5%, respectively, while after enzyme treatment, it decreased to 6.1 and 12.8%, respectively. Also, treating seeds with cell wall degrading enzymes facilitated the destruction of seed cell walls and increased the yield of oil during cold pressing of tomato and pomegranate seeds to 34.5 and 66.7%, respectively.

3.5.2. Nutritive value of tomato and pomegranate seed oils

The nutritive value of oils from tomato and pomegranate seeds was evaluated by determining the fatty-acid, microelement and vitamin composition.

The fat composition in fatty acids was determined by using methyl esters. Before GC-MS analysis, all samples were subjected to purification process consisting of: (a) purification of triglyceride fraction; (b) esterification with alcohol/NaOH and (c) derivation of the methyl esters to fix positions of unsaturations (oxazole derivatives). Whereupon, a 2 μl sample from each lot analyzed by chromato-mass-spectrometry method. The spectral data was compared to mass-spectral library "Wiley 275" and identified the following fatty-acids (Table 11).

The Table 11 shows tomato and pomegranate oil fatty-acid composition which were obtained after enzyme treatment in comparison with the control seed oils. The fatty-acid composition of oils which were obtained after enzyme processing remains unaltered but in both cases the content of unsaturated fatty acids were more than other vegetable oils. Tomato oil is especially rich in di- and tri-unsaturated fatty acids, while the pomegranate oil is rich in tri-unsaturated and linolenic acid. Also, a high content of linolenic acid in pomegranate seed oil was reported by Kohno et al [89] as well.

Microelement composition of tomato and pomegranate oils obtained by chemical extraction (benzene) as well as by enzyme treatment and cold pressing was determined by mass spectrometry using inductive-bound plasma method. The data in Table 12 shows the advantage of enzyme treatment and cold pressing method over chemical extraction.

For defining the biological property of oils, it is important to determine the composition of vitamins since they play an important role in human health.

Table 11. Fatty-acid composition of oil from tomato and pomegranate seeds (% total fatty acids).

N_0	Fatty acids	In tomato oil		In pomegranate oil	
		Before	after	before	after
1	n-Hexadecanoic acid (C16:0)	5.21	5.18	3.85	3.83
2	9-Hexadecanoic acid* ($C16:1^{\Delta 9}$)	2.46	2.46	5.76	5.91
3	n-Heptadecanoic acid (C17:0)	4.81	4.37	-	-
4	9,12-Octadecadienoic acid* ($C18:2^{\Delta 9,12}$)	16.87	16.69	7.72	7.74
5	n-Octadecanoic acid (C18:0)			2.33	2.38
6	9,12,15-Octadecatrienoic acid* ($C18:3^{\Delta 9,12,15}$)	26.51	26.54	33.19	33.25
7	7-Hexadecanoic acid* ($C16:1^{\Delta 7}$)	15.04	15.12	-	-
8	Linolenic acid (($C18:3^{\Delta 9,12,15}$)	8.09	7.88	32.21	31.98
9	16-Octadecanoic acid* ($C18:1^{\Delta 16}$)			3.25	3.28
10	13,16-Octadecadienoic acid* ($C18:2^{\Delta 13,16}$)	18.65	19.11	-	-
11	9-Octadecanoic acid* ($C18:1^{\Delta 9}$)			4.62	4.61
12	6,9,12-Octadecatrienoic acid* ($C18:3^{\Delta 6,9,12}$)			2.69	2.61

* - Unsaturated fatty-acids

Table 12. Comparative microelement composition of oil and oil cake from tomato and pomegranate seeds obtained by chemical extraction and cold pressing methods, mg/g oil.

Tomato oil							
	Mg	K	Ca	Fe	Mn	Zn	J
Extracted	0.0089	0.0542	0.0021	0.1702	0.00045	0.00055	0.00185
Cold pressed	**0.1234**	0.0637	**0.6481**	0.0349	**0.00237**	**0.00566**	**0.00511**
Pomegranate oil							
	Mg	K	Ca	Fe	Mn	Zn	J
Extracted	0.0046	0.0634	0.0134	0.1702	0.00171	0.00107	0.0094
Cold pressed	**0.0646**	0.0758	**0.4923**	**0.2974**	0.00195	**0.00324**	**0.0218**

The fat-soluble vitamins were analyzed by HPLC method using Hypersil ODS-C_{18}, 3 μm, (2x125 mm) at 50^0C with isocratic solvent (water and methanol mixture, 5:95%) and diode detector.

As shown in table 13, 1g pomegranate oil contains 26.1 and 155 mg of vitamin A (retinene) and E (ergo-tocopherol), while tomato oil contains only 1.45 mg of vitamin A (retinene). Also, both oil cakes contain vitamins A, C and E (Table 13). The capillary electrophoresis showed that both oil cakes contain

Table 13. Vitamin composition of oil and oil cake from tomato and pomegranate seeds before and after enzyme treatment.

	Tomato				Pomegranate			
	Oil		Oil cake		Oil		Oil cake	
Vitamins	(mg) per 1g oil		(mg) per 100g oil cake		(mg) per 1g oil		(mg) per 100g oil cake	
	before	after	before	after	before	after	before	After
Vitamin A	1.37	1.45	0.030	0.032	18.4	26.1	0.002	0.007
Vitamin C	-	-	27.31	27.5	-	-	39.9	54.01
Vitamin E	-	-	3.44	3.46	107.6	155	0.68	0.81
Vitamin B_1	-	-	2.32	3.47	-	-	-	-
Vitamin B_3	-	-	49.25	78.67	-	-	357.21	418.84
Vitamin B_6	-	-	6.36	9.98	-	-	-	-
Vitamin PP	-	-	7.04	7.72	-	-	6.98	7.23

vitamins B_1, B_3, B_6 and PP (Table 13). Thus, the oil and oil cakes of tomato and pomegranate seeds obtained by enzyme treatment followed by cold pressing did not have any negative effect on their food and biological value.

Conclusions

The tomato and pomegranate seed shells contained 15.3 and 18.7% of cellulose, 11.5 and 21.4% of lignin, 7.9 and 13.1% of readily hydrolysable polysaccharides (RHP) and 20.0 and 31.4% of non-readily hydrolysable polysaccharides (NRHP), respectively. Hydrochloro-acetic lignin from pomegranate seeds was with high molecular mass and a high degree of poly-dispersion capacity compared to that of tomato seeds.

Of the fungi screened, *Aspergillus terreus* produced the highest level of cellulase and xylanase, while *Pleurotus ostreatus* secreted the highest level of lignin-peroxidase. Two cellulases from *A. terreus*, a lignin-peroxidase from *P. ostreatus*, an endoglucanase and a xylanase from a psychrophilic fungus, *Aspergillus foetidus* and a xylanase and a polygalacturonase from a fungus *Cladosporium herbarum* were purified and characterized.

Fermentative studies revealed that the enzyme mixture from *A. terreus* (cellulases I and II), *A. foetidus* (endoglucanase and xylanase), *C. herbarum* (xylanase and polygalacturonase) and *P. ostreatus* (lignin peroxidase) at a ratio of 1:2:1:3 at pH 4.0 and at 40^0C was found to be ideal for optimal treatment of the isolated ligno-carbohydrate part from tomato and pomegranate seeds. This combination of enzyme mixture increased the degree of swelling, released high levels of reducing sugars and increased the weight loss and disintegrated the structure of tomato and pomegranate seed shells.

The main parameters that determined the cold pressing of seeds were humidity, pressure and temperature. By controlling the (a) humidity of seeds

before pressing, (b) temperature of seeds and oil, (c) oil cake and press forming matrix, and the (d) pressure through electronic motor, the conditions for extracting oil from seeds by cold pressing were optimized. Using the mini-press, oil from tomato and pomegranate seeds was extracted by cold pressing at temperatures below 40^0C with seeds having 8-10% humidity. Also, the mechanical grinding of seeds revealed that a particle size of 250 μm was optimal to extract maximum oil during cold pressing. The enzyme treatment of tomato and pomegranate seeds increased the oil yield by 34.5 and 66.7%, respectively.

A comparative study revealed that tomato and pomegranate seed oils obtained by cold pressing after enzyme treatment contained higher levels of micronutrients than that obtained by chemical extraction. Pomegranate seed oil contained 26.1 and 155 mg/g vitamins A and E, while tomato seed oil contained only 1.5 mg/g vitamin A. In contrast, the oil cake from two seeds contained appreciable amounts of vitamins A, C, E, B_1, B_3, B_6 and PP. Pomegranate oil cake was rich in vitamin B_3 (0.5g/100g).

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