World Academy of Science, Engineering and Technology
International Journal of Chemical and Molecular Engineering
Vol:5, No:8, 2011

Effect of Enzyme and Heat Pretreatment on Sunflower Oil Recovery Using Aqueous and Hexane Extractions

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***Abstract*—**The effects of enzyme action and heat pretreatment on oil extraction yield from sunflower kernels were analysed using hexane extraction with Soxhlet, and aqueous extraction with incubator shaker. Ground kernels of raw and heat treated kernels, each with and without Viscozyme treatment were used. Microscopic images of the kernels were taken to analyse the visible effects of each treatment on the cotyledon cell structure of the kernels. Heat pretreated kernels before both extraction processes produced enhanced oil extraction yields than the control, with steam explosion the most efficient. In hexane extraction, applying a combination of steam explosion and Viscozyme treatments to the kernels before the extraction gave the maximum oil extractable in 1 hour; while for aqueous extraction, raw kernels treated with Viscozyme gave the highest oil extraction yield. Remarkable cotyledon cell disruption was evident in kernels treated with Viscozyme; whereas steam explosion and conventional heat treated kernels had similar effects.

***Keywords*—**Enzyme-assisted aqueous and hexane extraction, heat pretreatment, sunflower cotyledon structure, sunflower oil extraction

I. Introduction

INDUSTRIAL processes for the recovery of the easily extractable oil from sunflower seeds is usually achieved by conventional mechanical pressing with expeller press, or through mechanical press followed by extraction with an organic solvent such as commercial grade hexane or ether [1], [2]. The following processing steps are usually employed, such as grinding and treatment by moisture with the reason of weakening the oil-bearing cell coats and preparing the seed for optimal oil extraction [3], [4]. However, although some of the oil content in sunflower seeds is easily extractable by simple use of conventional mechanical press or organic solvent, the rest of oil matter is strongly bonded to the seed matrix and extensive treatments are required in order to separate this fraction.

From reports it is possible to achieve oil yields in excess of 95% using conventional solvent-based processes [5], [6]. However, solvent extraction has disadvantages such as expensive operational costs and capital investment; poor quality of end-products due to the high processing temperatures; safety and environmental implications including plant safety problems, emissions of volatile organic compounds into the atmosphere and risks of fire and explosion in large installations [7], [8]. In 2001, the U.S. Environmental Protection Agency issued stringent guidelines for hexane emissions by vegetable oil extraction facilities [9]. As a result, the edible oil industry is looking for suitable and environmentally friendly methods of edible oil extraction [10].

Mechanical pressing processes are environmentally friendly and produce oil of high quality [11] as compared to solvent extraction. Some drawbacks of mechanical pressing include higher power consumption, high working costs, and lower oil recovery (yield) [12]. This has renewed interest in alternative oil extraction processes that may have less environmental effects as well as produce high and quality oil yield and also improve the potential application of the residual meal. Aqueous extraction processes are reported to represent a novelty in extraction technology for processing oilseeds. It represents no risks of fire and explosion, the solvent (water) is not toxic, ensures high quality products - oil and proteins [13], [14]. The main disadvantages compared to the conventional methods, are the lower efficiency of oil extraction yield, and the less stability of the product containing more residual oil [13].

Various studies suggest that enzymes can be used to enhance oil yield in oilseed extraction processes, by debilitating the oil-bearing seed cotyledon cell-wall, facilitating oil release prior to or during the oil extraction process. Enzyme pre-treatment before mechanical pressing for enhanced oil extractability has been reported for virgin-grade olive oil [15]), Guevina avellana mol [16], and rapeseed oil and sunflower oil [17], and cottonseed [18] using various enzyme mixtures containing pectinase, cellulase, and hemicellulase. Improved oil yield by some authors have been reported for enzyme-assisted solvent extraction processes. Enzyme pretreatment followed by solvent extraction has been reported for soybean oil [19], Ricinodendron heudelotii seeds oil [20], and sunflower seeds [21]. However, these studies did not investigate the possibility of achieving high oil recovery yield at shorter extraction time when the oilseeds are enzymatically and/or heat treated.

Studies on enzymatic aqueous extraction have been reported. Moreau *et al.* [22] applied different commercial cellulases in aqueous medium to to significantly increased oil extractability of corn oil, obtaining yields of about 80%. The use of enzymes for enhance oil recovery in aqueous vegetable oil extraction processes have also been reported for peanut

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[23], sunflower [24], [25], and canola [26], using various cell-wall degrading enzyme preparations.

The general structure of oilseeds has been reported by Rosenthal *et al*. [14], by dividing the seed cell wall structure into three basic domains, namely the Amphipathic domain at the amino terminus associated with the oil body surface; the Central hydrophobic domain containing non polar amino acids; and the Amphipathic domain at or near the carboxyl terminus surrounding the triacylglycerol matrix. However, their work did not emphasize on the effect of enzymatic action or heat pretreatment on the cell wall structure of oilseeds, especially sunflower seed.

Although various studies have reported improved oil recovery when the oilseeds are treated with enzymes, reports on the effect of heat treatment before or during enzymatic extraction processes on oil yield and seed cell wall structure of sunflower seed are not available. The objectives of this study therefore are to investigate the following effects on sunflower oil extraction yield: (i) enzyme action; (ii) heat pretreatment; (iii) combine heat pretreatment and enzyme action, all under conditions of hexane and aqueous extraction; and (iv) enzyme action, and heat pretreatment on the image of sunflower kernel cell structure. The work also examines the possibility of applying short extraction time in hexane extraction for improved oil yields.

II. MATERIALS AND METHODS

A. Raw Materials

Sunflower (*Helianthus annuus L.*) kernels (i.e. dehulled seeds) were obtained from Healthy Living, Loughborough, UK. The kernels were cleaned to remove foreign materials such as stones, dirt and undeveloped kernels.

A commercial *Aspergillus aculeatus* formulation, "Viscozyme L" was obtained from Novozymes (Bagscaerd, Denmark). Viscozyme L is a multi-enzyme complex containing a mixture of carbohydrases; including arabinase, cellulase, β-glucanase, hemicellulase and xylanase, with some activity on branched pectin substances.

B. Heat Pretreatment of Kernels

The kernels were heated using a laboratory autoclave (Astell Scientific Portaclave AAJ04, UK) at 120^{o}C and 1 bar for 30 minutes. For "steam explosion", the pressure was suddenly released from 1 bar to zero (0) within 5 s. For "conventional heat treatment", the pressure was allowed to fall gradually till it reached zero (0). The heat treated kernels were stored in a desiccator.

C. Enzyme Pretreatment of Kernels

Two millilitres (2 ml) of "Viscozyme L" was added to 100 ml of citrate buffer (pH 5.0) in a beaker followed by 20 g of raw sunflower kernels, and then placed in a water bath at 50^{o}C for 24 hours. The kernels were then transferred into a beaker and carefully dried in an electric oven (IM-30, Irmeco, Germany) at 105 ± 1^{o}C for 50 minutes to reduce the kernel moisture content. The dried kernels were covered tightly and placed in a desiccator.

Similarly, 20 g each of previously steam exploded and conventional heat treated kernels were enzyme treated as above. Also, additional samples of raw, conventional heat treated and steam exploded kernels were treated with Viscozyme, dried and kept in a desiccator.

D. Enzyme-Assisted Hexane Extraction

Raw sunflower kernels treated with "Viscozyme L" were grinded with mortar and pestle, and sieved to the particle size between 0.762 mm and 1 mm (optimum particle size as observed by Dominguez *et al.* [24] and Seneiro *et al.* [25] and), which favour the enzymatic action and permit free flow of the solvent for efficient extraction.

Ten grams (10 g) of the ground kernels were placed in a Soxhlet apparatus with hexane (99% HPLC grade) at 60 ^{o}C for 1 hr; and the miscella evaporated to obtain the extracted oil. The mass of the extracted oil was calculated as the weight differences between empty flask and the flask with oil. The percent oil extracted was estimated as the mass of the oil extracted divided by the mass of the kernel sample used multiplied by 100. Also, the percent total extractable oil was calculated by dividing the percentage oil extracted by the percent total oil extractable from sunflower, and multiplied by 100. Triplicate determination was performed.

Similarly, oil was extracted in triplicates, each from a previously steam exploded and conventional heat treated sunflower kernels without "Viscozyme L" treatments. The percentage total oil extractable was again calculated in each case. Also, oil extraction was performed with steam exploded and conventional heat treated kernels additionally treated with "Viscozyme L" (in three replicates) for 1 hr. Extraction of two control samples (raw kernels) were carried out in triplicates, firstly using 1 hr as above, and also for 4 hrs maximum time.

E. Enzyme-Assisted Aqueous Extraction

A modified method by Seneiro *et al.* [25] was used. Twenty grams (20 g) of the ground raw kernels of particles between 1 and 0.762 mm were placed in a beaker containing 120 ml of a 0.05 M citrate buffer solution at pH 5.0 (the optimum pH for maximum activity of "Viscozyme L" recommended by the manufacturer). This was taken as the control. Exactly 2 ml of "Viscozyme L" enzyme preparation was added to a second sample containing 120 ml of 0.05 M citrate buffer solution at pH 5.0 on 20 g ground raw kernels in a beaker. Similarly, 2 ml of "Viscozyme L" was added to samples of previously conventional heat treated and steam exploded kernels in separate beakers. A total of six (6) separate samples were prepared for the aqueous extraction. Also, 120 ml of the 0.05 M citrate buffer solution at pH 5.0 was added to 20 g samples each of previously conventional heat treated and steam exploded kernels without addition of any "Viscozyme L".

The six (6) suspensions were then incubated and mixed at 150 rpm in an incubator (Stuart Scientific Orbital Incubator S-150, UK) at 50^{o}C (the optimum temperature to favour the activity and stability of the enzyme recommended by the manufacturer) for 2½ hrs. The two phases (solid/liquid) were separated by centrifugation at 11,000 rpm at 4 ^{o}C for 20

minutes using a laboratory centrifuge (Labortechnik HERMLE Z-383K, Germany) to recover the oil. The oil obtained was weighed.

The solid residue obtained from the centrifugal process was dried and the residual oil extracted using hexane with a Soxhlet. The oil recovered from the liquid phase after the centrifugation process was calculated as the difference between the total oil content in the kernels and the residual in the solid product. The percentage total extractable oil was calculated as the percentage of the oil content in the liquid phase on the total oil present in the sunflower kernels or seeds. Triplicate determination was performed.

F. Seed Imaging Analysis

Sunflower kernels (i.e. dehulled seeds) under different conditions (raw, conventional heat treated, steam exploded and enzyme treated as outlines above) were microscopically examined to determine the visible effects of the various treatments on the kernel cotyledon/cell structure. The kernel samples were cut with razor blade and a fixative solution was added to it; containing 1 ml 25% gluteraldehyde, 2.7 ml formaldehyde, 2.5 ml of 0.05 M sodium phosphate buffer of pH 7.2, 18.8 ml deionised water, 25 ml Triton X-100 and fixed overnight at 4^{o}C. The samples were then washed at 1 hr intervals for 3 times with 0.05 M sodium phosphate buffer of pH 7.2, and dehydrated by adding 30, 50, and 70% ethanol solutions at 2 hrs intervals and kept overnight at 4^{o}C. Before each addition, the old solution was taken out using fresh Pasteur pipettes. More concentrated ethanol solutions of 90 and 100% were added and kept for 2 hrs respectively.

The samples were infiltrated by adding 100% ethanol and Histoclear solutions and kept for 1 hour followed by addition of 20 pellets of paraffin wax and kept overnight at room temperature. After 24 hrs, additional 20 pellets were added, every hour for 5 hrs (replacing the old with fresh ones) at 42^{o}C to saturate the samples. Again, another 20 pellets were added at 60^{o}C for 1 hr, 3 hrs, and 48 hrs – (while changing the paraffin every 24 hrs) respectively.

The samples were placed into embedded moulds, vertical to the base, and brought to room temperature to set. Sections of 5-7μm thick were cut using a wax microtome (MICROM International Rotary Microtome HM-315, Germany). The ribbons were floated on a slide with water and placed on a hot plate and left to dry. The slides were incubated overnight at 37^{o}C, with the addition of ethanol, histoclear, SDW and toluidine blue (as the stain) after 24 hours at 1 minute intervals each. The slides were flooded with water, and as soon as the water douches the first section, DePex was put on a coverslip, placed over sections and allowed to dry over night. The slides with sections were placed on a microscope (Nikon Optiphot OPTIPHOT-2, Japan), and images taken and recorded.

III. RESULTS AND DISCUSSIONS

A. Effect of Enzyme and Heat Pretreatment on Oil Yield during Hexane Extraction

A graph of percent oil extraction yields versus seed samples for raw, and various heat and enzyme treated sunflower kernels is shown in Fig. 1. Generally, enhanced oil extractable were obtained for both heat pretreated kernels either with or without 'Viscozyme L', and raw kernels pretreated with 'Viscozyme L', than the raw (untreated) kernels extracted in 1 hour.

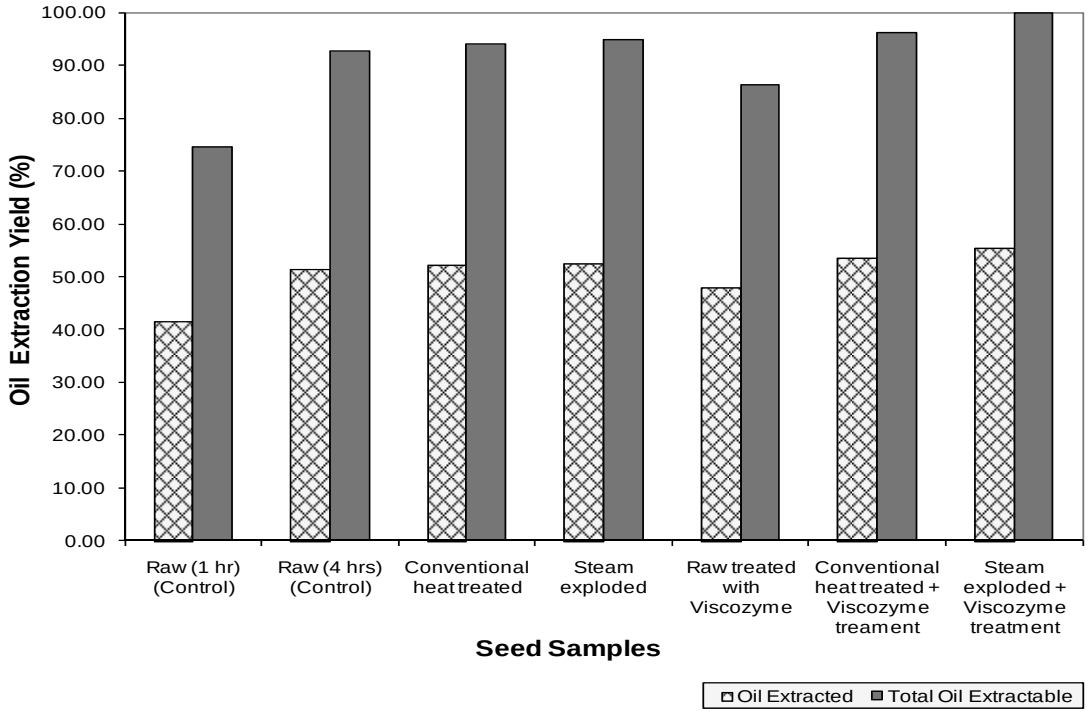

Fig. 1 Percent oil extraction yields for raw, heat treated and "Viscozyme" treated sunflower kernels before hexane extraction with Soxhlet

Oil extraction yield of 51.23%, that is 92.73% of the total extractable oil and 41.36% representing 74.66% of the total extractable oil were obtained for the raw kernels, taken as the control at 4 hours and 1 hour respectively. It was observed that, for all the treated sunflower kernels, only raw kernels treated with 'Viscozyme L' before the extraction gave lower extracted oil than the control extracted in 4 hours maximum time. However, the oil yield of 47.78% indicating 86.25% of the total extractable oil obtained from the raw kernels pretreated with 'Viscozyme L' was higher than 41.36%, signifying 74.66% of the total extractable oil obtained for the control at the same 1 hour extraction time. This represents 11.59% of the total extractable oil, higher than the control in 1 hour extraction time. This was an enhanced oil yield than 4.00% increased of extractable oil reported by Dominguez *et al.* [21], for extraction of sunflower oil with different commercial enzymes at maximum extraction time. The improved oil recovery obtained from the raw kernels treated with 'Viscozyme L' than the corresponding control extracted in 1 hr was due to the more efficient circulation of the solvent as a result of the increased in the permeability of the cell wall caused by the removal of the pectin layer, and disruption of the cotyledon cell and bonding membranes of the kernels by the enzyme (see Fig. 2).

1. Effect of Heat Pretreatment of Kernels on Oil Yield

Oil yields obtained from conventional heat treated and steam exploded sunflower kernels before the solvent extraction were higher than both control samples. Extracted oil of 52.02%, that is 93.90% of the total extractable oil was obtained from conventional heat treated kernels; whiles 52.48% extracted oil representing 94.73% of the total extractable oil was obtained from steam exploded kernels. The enhanced oil extractable obtained in each case than the control, and even raw kernels treated with "Viscozyme L" were because the cotyledon containing the cytoplasm network of the kernel cell wall were denatured and broken with oil bodies loosened and released as results of the heating. Also, both preheating methods of the kernels caused coagulation of the protein bodies, and thus enhance the released of oil from the lipid bodies. Evidence of the kernel cell wall disruptions are shown in Fig. 2.

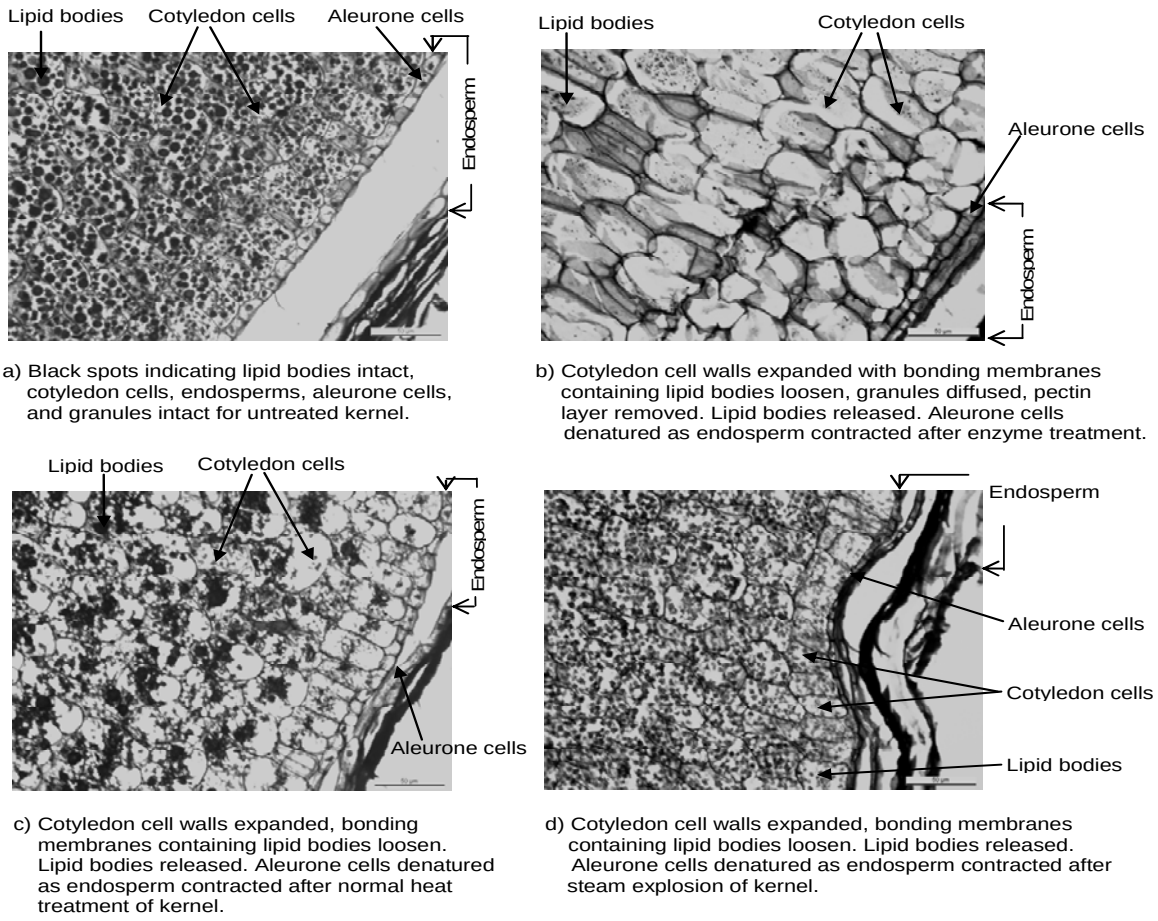

Fig. 2 Sections of imbedded kernel of sunflower stained with Toluidine blue showing various lipid bodies in cells of the cotyledon in the extreme side of the kernel (magnification 20x): (a) raw kernel, (b) raw kernel treated with "Viscozyme" L, (c) conventional heat treated kernel, (d) steam exploded kernel.

2. *Combined Effects of Enzyme and Heat Pretreatment of Kernels on Oil Yield*

With further 'Viscozyme L' (enzyme) treatment of conventional heat treated and steam exploded kernels before the extraction, the highest yields were obtained. Enhanced oil extraction yield of 53.33% indicating 96.26% of the total extractable oil was obtained for conventional heat treated kernels with further treatment with 'Viscozyme L', and 55.38% extracted oil, that's is 99.96% of the total oil extractable was obtained for steam exploded kernels additionally treated with 'Viscozyme L'. The enhanced oil efficiency obtained was due to additional breaking of the cotyledon cells making the oils bodies more released and the kernels structure more permeable, thus allowing more efficient percolation of the hexane and consequently enhancing liberation of the oil. (Images not included.)

Comparatively, from Fig. 2, image of the raw kernel ('a') showed intact structured network of aleurone grains, endosperm, and cotyledon within which contained cytoplasm network consisting of proteins and lipid bodies. The oil/lipid bodies indicated by black spots were intact in the cotyledon. The cell content was surrounded by a rather thick wall which has to be opened so that the oil can be released. For the raw kernels treated with 'Viscozyme L' ('b'), the pectin layer was removed by the enzymatic action; granules diffused, cotyledon/cell wall expanded, bonding membranes loosen with oil bodies released. Conventional heat treated kernels ('c'), and steam exploded kernels ('d') exhibited identical influence on the cell wall structure. The cotyledon consisting of the cytoplasm network was denatured and aleurone cells in the endosperm denatured as endosperm was contracted. However, the cotyledon cells of the steam exploded kernels look comparatively more damage. This was as a result, the reason why more oil yield was obtained from steam exploded kernels than the conventional heat treated kernels.

B. Effect of Enzyme and Heat Pretreatment on Oil Yield during Aqueous Extraction

Results for extracted oil obtained from raw (control), conventional heat treated, steam exploded, and 'Viscozyme L' (enzyme) treated sunflower kernels are shown in Fig. 3. The extraction yield of the control was 15.95 %, which represented 28.77% of the total extractable with Soxhlet. Oil extractable yields of about 30–40% of the total extractable oil are usually reported for aqueous extraction of ground sunflower (< 1 mm) followed by batch centrifugal separation of the solid and liquid phases [24], [25].

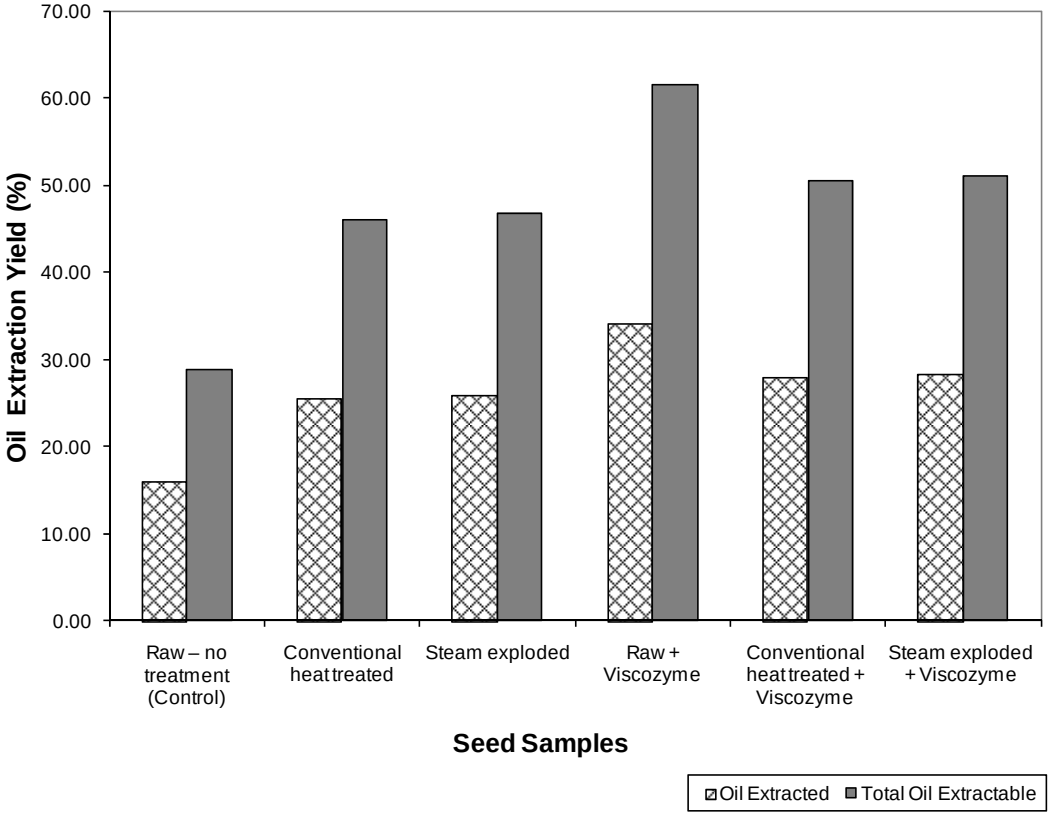

Fig. 3 Percent oil extraction yields for raw, heat treated and "Viscozyme" treated sunflower kernels in aqueous extraction

Oil yields of between 25.51% that represented 46.05% of the total extractable, and 25.88% indicating 46.72% of the total extractable were obtained from previously conventional heat treated and steam exploded sunflower kernels, respectively. These correspond to improved total extractable yields of 17.28 and 17.95% for conventional heat treated and steam exploded kernels respectively compared to the control. The increased in oil yields were due to the fact that the cytoplasm contents of the heat treated kernels were broken enhancing the release of oil from the cytoplasmic cells as the inner structure is loosened as compared to the raw kernels with intact cytoplasmic network. This is shown in Fig. 2 ('a'), ('c'), and ('d').

From the results obtained, raw sunflower kernels hydrolysed with 'Viscozyme L' was found to be the most efficient, producing oil yield of 34.05%, which represented 61.46% of the total extractable oil. This was higher than the 30% of total extractable oil reported by Dominguez *et al.* [24], and Sineiro *et al.* [25] using mixtures of Cellulase and Pectinase.

1. *Combined Effects of Heat Pretreatment and Enzyme Action on Oil Yield*

Comparatively, oil yields of 27.96% precisely 50.47% of the total extractable, and 28.29% that is 51.07% of the total extractable oil were obtained for conventional heat treated and steam exploded sunflower kernels further hydrolysed with 'Viscozyme L' respectively. Surprisingly, although only heat pretreatment of the kernels before the aqueous extraction without enzymatic hydrolysis resulted in higher oil yields than the control, the oil extractable yields obtained from the combined effects of the enzyme hydrolysis and heat pretreatment of the kernels were lesser than the 61.46% of total extractable oil obtained from the raw (non-heat pretreated) kernels hydrolysed with the enzyme. This was because the longer hydrolysis time for the combined heat pretreatment and enzyme treatment may have caused soluble sugar caramelisation, enzyme inactivation by temperature and also decoagulation of protein bodies that consequently reduced the oil extraction yield.

The enhanced oil extractability obtained from the raw (non-heat pretreated) kernels hydrolysed with 'Viscozyme L' indicated that the structure of the cotyledon cell walls of the kernels were broken and slackened, which made the cell wall structure more permeable, and thus improving the release of oil as compared to all the other samples. The effect of the enzyme action on the cytoplasm structure of the raw sunflower kernels treated with 'Viscozyme L' is shown in Fig. 2 (b). From the microscopic image, the granules were diffused; pectin layer removed, bonding membranes and walls loosen with the cell walls expanded.

B. Comparison of the Aqueous and Hexane Extractions

As shown in Fig. 1 and Fig. 3, the hexane extraction produced higher oil yields than the aqueous extraction for all samples of sunflower kernels used. During the hexane extraction, higher oil recovery were obtained from the conventional heat treated, and steam exploded kernels than the raw kernels treated with 'Viscozyme L', while in the aqueous extraction the raw kernels treated with 'Viscozyme L' produced the highest oil recovery. In the hexane extraction the maximum oil recovery was obtained from the combined treatments of steam explosion and 'Viscozyme L' to the sunflower kernels before the extraction; and the nearest oil recovery obtained from the conventional heat treated kernels with further 'Viscozyme L' treatment. However, in the aqueous extraction, the combined application of steam explosion, and conventional heat treatment with 'Viscozyme L' produce any encouraging oil yields, with oil recovery significantly lesser than that obtained from the raw kernels treated with 'Viscozyme L' only.

IV. Conclusion

The study investigates the effect of enzyme action, heat pretreatment, combine heat pretreatment and enzyme action on sunflower oil extraction recoveries using hexane and aqueous extraction processes, as well as on the sunflower kernel cell structure. The study showed that heating sunflower kernels before hexane and aqueous extraction schemes helped to obtain improved oil recovery. For maximum oil extractable, the combined application of steam explosion and "Viscozyme L" treatment of the kernels before hexane extraction was the most efficient even in a short period of 1hr, whereas in the aqueous extraction applying "Viscozyme L" treatment alone to raw the kernels gave the highest oil recovery. Thus, for maximum oil recovery, the combined effects of heat and enzyme treatments favour hexane extraction but not the aqueous extraction. The study also found that preheating and treating sunflower kernels with 'Viscozyme L' both break down the cytoplasmic cells of the kernels and caused loosen of the inner cotyledon structure, though the enzyme action additionally caused the removal of the pectin layer, diffused of granules and expansion of the cotyledon cells.

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