

## Enzyme-assisted three-phase partitioning: An efficient alternative for oil extraction from Sesame (*Sesamum indicum* L.)

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**SUMMARY:** Three-phase partitioning (TPP) was explored for oil extraction from *Sesamum indicum* L. seeds. The process parameters, namely the salt concentration, slurry/*t*-butanol ratio and system pH were standardized. The optimum conditions for maximum oil recovery using TPP were an ammonium sulphate concentration of 40% (w/v), slurry/*t*-butanol ratio of 1:1 (v/v) and system pH of 5.0. The powdered seeds were subjected to enzyme-assisted three-phase partitioning (EATPP) which was pre-treated with pectinase, protease and a mixture of  $\alpha$ -amylase and amylo-glucosidase (1:1 ratio) followed by TPP (as standardized conditions) and its efficacy in recovering oil was compared with TPP and solvent extraction (SE). Out of all the enzymes studied, EATPP with pectinase resulted in the highest oil recovery (86.12%), which was higher than that of TPP (78.24%). The free fatty acids, saponification value and peroxide values were observed to be lower in the case of TPP and EATPP when compared to SE, indicating better oil quality.

**KEYWORDS:** Cell wall hydrolysis; Enzyme-assisted three-phase partitioning (EATPP); Solvent extraction (SE); Green extraction method; Sesame oil

**RESUMEN:** *Partición trifásica asistida por enzimas: una alternativa eficiente para la extracción de aceite de sésamo (Sesamum indicum L.).* Se investigó la partición trifásica (TPP) para la extracción de aceite de semillas de *Sesamum indicum* L. Los parámetros del proceso, como la concentración de sal, la proporción de suspensión/*t*-butanol y el pH del sistema, fueron estandarizados. Las condiciones óptimas para la recuperación máxima de aceite utilizando TPP fueron una concentración de sulfato de amonio del 40% (p/v), una relación de suspensión/*t*-butanol de 1: 1 (v/v) y un pH del sistema de 5.0. Las semillas en polvo se sometieron a una partición trifásica asistida por enzimas (EATPP) que se trata previamente con pectinasa, proteasa y mezcla de  $\alpha$ -amilasa y amilo-glucosidasa (relación 1:1) seguida de TPP (como condiciones estandarizadas) y su eficacia como recuperación de aceite se compara con TPP y extracción con solventes (SE). De todas las enzimas estudiadas, la EATPP con pectinasa produjo la mayor recuperación de aceite (86.12%), que es mayor que la de TPP (78.24%). Los ácidos grasos libres, el índice de saponificación y de peróxidos fueron más bajos en el caso de TPP y EATPP en comparación con SE lo que indica mejor calidad del aceite.

**PALABRAS CLAVE:** Aceite de sésamo; Hidrólisis de la pared celular; Método de extracción verde; Partición trifásica asistida por enzimas (EATPP); extracción por solvente (SE)

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## 1. INTRODUCTION

The sesame (*Sesamum indicum* L.) seed, known to mankind for about 6000 years, is a source of oil (45-55%), protein (20-25%) and other bioactive constituents like sesamol, sesamin, sesamolins etc. which act as natural antioxidants (Namiki, 2007). Sesame seeds and their oil have unique physiological and nutritional properties which have extensive applications in the field of cosmetology and medicine. Today, apart from their medicinal use, sesame seed powder and oil are widely used in confectionery and baked products (Namiki, 1995). Sesame oil is mainly extracted by expeller pressing, followed by non-polar solvent extraction. Expeller processing involves high heat treatment which adversely affects the oil quality besides denaturing the protein (Latif and Anwar, 2011).

In the case of solvent extraction (SE), hexane is the most commonly used solvent for oilseed extraction owing to its convenience, non-corrosive nature and low cost (Panadare and Rathod, 2017). However, hexane is known to contain a light petroleum fraction and is characterized by a narrow boiling range and high flammability. Hexane, when released into the environment, may participate in the formation of photo-chemical smog in the atmosphere, and is therefore, considered as a pollutant that has adverse effects on human health (Ferreira-Dias *et al.*, 2003; Vidhate and Singhal, 2013; Tan *et al.*, 2016). In spite of the known hazards associated with hexane, large scale oil extraction plants continue to rely on it because alternative technologies that are competent at industrial scale do not exist.

In recent years, a rise in the research pertinent to novel, green oil extraction methods for edible and non-edible applications has been seen. One such method is three-phase partitioning (TPP), which is a potential alternative to the solvent (hexane) extraction that facilitates extraction of oil in addition to other bio-active compounds such as a protein from oilseeds (Sharma and Gupta, 2001a; Ruchi *et al.*, 2007; Ketnawa *et al.*, 2014; Mondal, 2015; Panadare and Rathod, 2017). TPP has recently been reported to be applied for oil extraction from various plant materials like flax seed (Tan *et al.*, 2016), mango kernels, rice bran, soybeans (Ruchi *et al.*, 2007; Sharama *et al.*, 2002), kokum (*Garcinia indica*) kernels (Vidhate and Singhal, 2013) and Jatropha seed kernels (Dutta *et al.*, 2015).

Even though TPP is a novel technique, it entails the drawback of relatively lower oil recovery compared to conventional solvent extraction. This drawback can be overcome by including enzyme-assisted pre-treatment techniques, which rupture the cell wall of oilseeds by hydrolyzing the polysaccharides and the proteins in the cell and membranes (Sosulski *et al.*, 1988).

Accordingly, the objective of this work was to standardize the operating conditions of TPP (such

as salt concentration, slurry/*t*-butanol ratio and pH) and to explore the process intensification of sesame oil recovery by EATPP. Attempts are made to achieve similar results to those of SE through eco-friendly methods like TPP and EATPP. The recovery and quality characteristics (physico-chemical and thermal) of the oil recovered by EATPP were compared with those of TPP and solvent-based extraction methods.

## 2. MATERIALS AND METHODS

Sesame (*Sesamum indicum* L.) seeds were obtained from a local store (Mysore, Karnataka, India). Ammonium sulphate, *t*-butanol and pectinase (3.5 U/mg, Cat no 90464) were acquired from Sisco Research Laboratories, Mumbai. Enzymes, such as  $\alpha$ -amylase (32.4 U/mg, Cat No. 10065), amylo-glucosidase (70 U/mg, Cat no. 10115) and protease ( $\geq 500$  U/g, Cat no.6110) were purchased from Sigma Aldrich, Germany. All chemicals and reagents used for analysis were of analytical grade.

### 2.1. Solvent extraction (SE) for estimating total oil content

The total oil content of sesame seeds was estimated by conventional solvent extraction (SE) using the Soxhlet method (AOAC, 2002). The oil was extracted from a finely ground Sesame seed sample using *n*-hexane by a Soxhlet extractor for 6 h at  $70 \pm 2^\circ\text{C}$ . After SE, the solvent was evaporated using a rotary evaporator at  $40^\circ\text{C}$ .

The oil obtained by solvent extraction was considered as reference (100%) to find out the recovery of the two methods, TPP and EATPP. The overall work plan is schematically presented in Figure 1.

### 2.2. Three-phase partitioning (TPP)

Finely ground Sesame seeds (5 g) were mixed in double-distilled water (25 mL) and used for the standardization of process parameters (salt concentration, slurry/*t*-butanol ratio and system pH) of TPP. The mixture was stirred gently on a stirrer and the pH was adjusted to the desired range of 3-9 (with an increment of 2), using NaOH (0.5 M) and HCl (0.5 M). The appropriate ammonium sulphate concentration (20-60% w/v) was added and stirred (Heidolph, RZR-2020, Germany) at 130 rpm for 15 min, followed by the addition of a pre-determined amount of *t*-butanol (1:0.5-1:3). The mixture was kept for 1.5h at ambient temperature ( $27 \pm 2^\circ\text{C}$ ) followed by centrifugation for 10 min (at 2000g) for phase formation. The top phase was separated in a flask and *t*-butanol was separated from the oil under reduced pressure and temperature using a rotary evaporator (Buchi V-850, Switzerland). The oil obtained was quantified and % recovery was

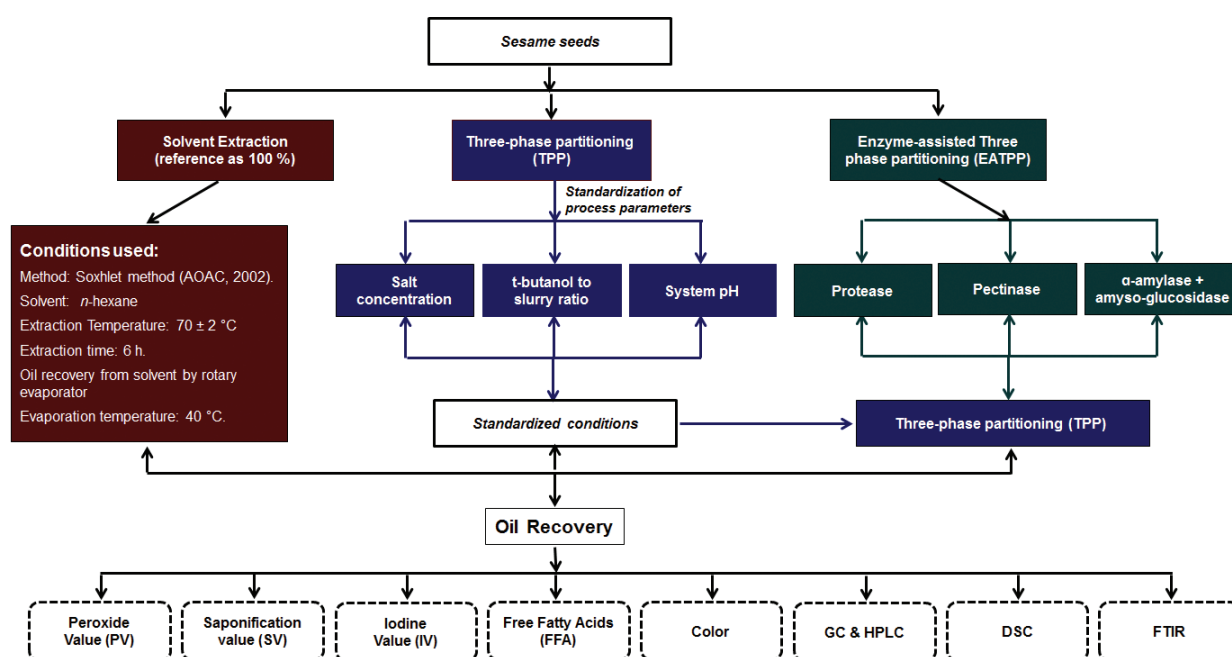


FIGURE 1. Overall work plan.

calculated on the basis of total oil content of the sesame seed.

### 2.3. Enzyme-assisted three-phase partitioning (EATPP)

The finely ground Sesame seed powder was subjected to EATPP by pre-treating the samples with enzymes followed by TPP. The pre-treatment was carried out with a pre-determined amount (5% by seed weight) of each of the three enzyme (pectinase,  $\alpha$ -amylase: amylo-glucosidase, 1:1 and protease) preparations at pH 4.0, 6.8 and 6.8 (optimum pH of respective enzymes), respectively. The slurry was incubated at  $40 \pm 2$  °C in a shaker incubator (Remi, CIS-24PLUS) with uniform stirring (50 rpm) for 60 min. The slurries obtained after incubation were subjected to TPP under standardized conditions after adjusting the pH (standardized for TPP).

### 2.4. Peroxide value

“The peroxide value (PV) is the milli-equivalents of peroxides present in 1000 g of oil or fat that oxidizes potassium iodide under suitable conditions” (Rossell and Pritchard, 1991). These values were measured according to the AOAC (2005) method. An oil sample (2 g) was dissolved in chloroform and acetic acid solution and saturated potassium iodide were then added to the sample. The peroxides present in the oil underwent oxidation, resulting in the release of iodine. The amount of iodine released (from potassium iodide) was determined by

titration with sodium-thiosulphate (0.1 N) using a starch ( $\text{sol}^n$ ) indicator.

### 2.5. Saponification value

The saponification values (SV) of the extracted oils (from EATPP, TPP and SE) were determined using the method prescribed by Sheema *et al.*, (2016). An oil sample (2 g) was boiled in a reflux-condenser (for 60 min) after adding 0.5 N alcoholic potassium hydroxide. The mixture was brought to ambient temperature and saponification values were estimated (expressed as ‘mg’ of KOH required to saponify per ‘g’ of oil, mg KOH/g) by titrating with 0.5N HCl.

### 2.6. Iodine value

The iodine value (IV) was estimated (AOAC,2005 method) for the oil obtained from EATPP, TPP and SE by adding Wijs solution to the oil sample. The sample was incubated (for 60 min) in dark conditions at ambient temperature ( $27 \pm 2$  °C). The mixture was then titrated with sodium thiosulfate using a starch solution as indicator. The iodine value was expressed as ‘g’ of iodine absorbed by 100 g of the fats.

### 2.7. Free fatty acids (FFA)

The methodology prescribed by Raja Rajan and Gopala Krishna (2014) was followed for the FFA analysis of the oils obtained from SE, TPP and

EATPP. The oil samples were boiled in the presence of neutralized alcohol and were allowed to cool at room temperature. The samples were then titrated with a NaOH solution using phenolphthalein as indicator. The FFA values were expressed as % oleic acid.

## 2.8. Measurement of color

The color of the sesame oil samples extracted by SE, TPP and EATPP was measured by transmission measurement in a 1-inch cell using a Lovibond tintometer color measurement apparatus (The Tintometer Ltd., Model-F, Salisbury, United Kingdom) and calculated as [(5 × Red units) + (1 × Yellow units)]. The results are expressed as Lovibond units (Raja Rajan and Gopala Krishna, 2014).

## 2.9. Gas chromatography (GC) analysis

The fatty acid composition of the oils obtained by SE, TPP and EATPP were analyzed using gas chromatography and the AOCS (2002) method was followed. Hexane (5 mL) and 2 N methanolic KOH (0.1 mL) were added to the oil (200 mg) and vortexed. The samples were heated on a water bath for a few minutes. The sample was then allowed to settle overnight, and the supernatant was then taken and analyzed for different fatty acids using GC (Shimadzu GC-2010 Plus-02, Kyoto, Japan) equipped with a flame ionization detector (FID) and fitted with a gas chromatography column Rtx-2330 (90% *bis*-cyanopropyl/10% phenyl cyanopropyl polysiloxane). The operating conditions of GC were injector pressure 107.1 kPa, injector temperature 250 °C, column temperature 260 °C, detector temperature 260 °C with a nitrogen, hydrogen and air flow rate of 30, 40 and 400 mL/min, respectively.

## 2.10. High-performance liquid chromatography (HPLC) analysis

The detailed procedure of the method used for the quantification of triacylglycerol molecular species present in oils is reported elsewhere (Debnath *et al.*, 2011). The same procedure was followed for the estimation of triacylglycerol molecular species in the oil extracted from SE, TPP and EATPP. The operating conditions used were Symmetry® RP-C18 (4.6 × 250 mm, 5 μm) column, mobile phase (acetone–acetonitrile ratio of 63.5:36.5, v/v), flow rate 1 mL/min. About 20 μl sample (20%, w/v, oil in chloroform) were injected for analysis.

## 2.11. Differential scanning calorimeter (DSC) analysis

The melting profiles of the oils extracted from SE, TPP and EATPP were studied using a DSC (Perkin Elmer, USA) equipped with a liquid nitrogen unit

(auto-cool accessory) for cooling DSC cells at a pre-determined rate. The nitrogen gas flow rate was kept constant at 10 mL/min (to avoid heat currents). The oil sample (5–10 mg) was placed in an aluminium pan (a hermetically sealed empty pan acted as the reference) and the temperature and heat flow data were recorded by the system software. The oil samples were initially heated rapidly (10 °C/min) to 80 ± 2 °C. The mixture was incubated (for 10 min) at this temperature to erase crystal memory followed by cooling at the rate of 10 °C/min to -40 °C. The mixture was left to rest for 3 min at this temperature. The sample was heated again at the same rate (10 °C/min) to 80 °C (AOCS, 2000). The heating/cooling thermograms recorded were used for the determination of temperature for the onset, completion and melting peak and enthalpy of melting.

## 2.12. Fourier transform-infrared spectroscopy (FTIR) analysis

The oil samples extracted from TPP and EATPP were analyzed by FTIR spectroscopy and compared with a *t*-butanol standard to detect its presence in the oil. FTIR spectra were recorded in the range of 3500–500 cm<sup>-1</sup> (Henna and Tan, 2009). All the data were recorded and integrated using the Nicolet Omnic 6® software.

## 2.13. Statistical analysis

The experiments were performed in triplicate (n=3). Means and standard deviations for three independent experiments and values are expressed as mean ± standard deviation (SD). The data were tested to determine significant variations among extraction methods through the application of one way ANOVA followed by post-Tukey multiple comparison test with 95% confidence level (p < 0.05) using Microsoft Excel-2013® and GraphPad Prism® software (Version 5.0, GraphPad Software, San Diego, CA).

# 3. RESULTS AND DISCUSSION

## 3.1. Estimation of total oil content

The estimation of total oil content was carried out by solvent extraction. It is a known fact that recovery from solvent extraction can be the highest that one can achieve and the objective of the work was to achieve comparable results of that of solvent extraction by TPP and EATPP by considering solvent extraction results as reference.

The conventional solvent based extraction (hexane) of sesame (*Sesamum indicum* L.) oil resulted in total oil content of 44.6 g/100g (w/w), which is taken as the reference value (100%) for determining the oil recovery achieved by other methods (TPP and EATPP). Sesame seeds are reported to have 45-50%

(w/w) of oil (Reshma *et al.*, 2010) and the observed results are in line with the reported literature.

### 3.2. Three-phase partitioning (TPP)

TPP involves the addition of phase-forming components such as salt and *t*-butanol to oil seed powder in an aqueous media followed by mixing. After incubation, the salting out of the proteins occurs from the saline aqueous medium to *t*-butanol. This phenomenon is an outcome of the presence of high sulphate ion concentration in the aqueous medium and its kosmotropic action, which is further facilitated by the presence of *t*-butanol. Subsequently, the separation of the three phases is carried out by centrifugation. Following centrifugation, the aqueous phase with carbohydrates, soluble fibers, salts etc. (water-soluble components) is obtained in the lower dense layer, the proteins float in the middle (medium-dense phase) and oil dissolved in *t*-butanol can be recovered from the upper-most layer also called the oil-rich phase (Ruchi *et al.*, 2007; Kurmudle *et al.*, 2011).

In this study we considered phase-forming salt concentration (w/w), slurry/solvent ratio (v/v) and pH as the process parameters for the standardization of TPP for improving the recovery of oil. The results of the effect of these process parameters are discussed in detail in the subsequent sections.

#### 3.2.1. Effect of salt concentration

The effect of salt concentration on oil recovery was studied and the results are shown in Figure 2a. As can be seen from this figure, the recovery of oil was found to increase with an increase in ammonium sulphate concentration from 20 to 40% (w/v). An increase in salt concentration causes osmolysis of cells, resulting in the rupture of the cells release of cytosolic material into the solvent (water) (Li *et al.*, 2015). As a result, vacuoles (oil bodies, prime location of oil in the cell) located in the cytosol of cells come into direct contact with the salt environment. The phospholipid groups of oil bodies are hydrophilic, which contain proteins infused into them. A portion of (~50%) these proteins is exposed to the salt environment and undergoes precipitation resulting in the release of triglycerides.

The complete solubilization of proteins in the oil body membrane causes its rupture (Vidhate and Singhal 2013; Dutta *et al.*, 2015). The high amount of precipitate (because of the presence of salt in the phase results in protein-protein interactions) formed is also an indication of better rupture of cell bodies, and, in turn, increased oil recovery.

The oil recovery increased with an increase in salt concentration from 20 to 40%, above which the yield/recovery decreased, resulting in a maximum at 40% salt concentration. The maximum oil recovery of 73.59%, w/w was achieved at 40% (w/v) ammonium

sulphate. Decreased recovery at higher concentrations of salt can be attributed to the altered phase composition. It appears that increasing the volume of the middle layer (because of the increased precipitation of proteins) results in changes in the phase composition (decrease in *t*-butanol concentration) of the top phase. As a result, the solvent content required for the solubilization of oil decreases, resulting in reduced oil recovery. In the literature, where TPP was used for the extraction of oil, it is reported that ammonium sulphate concentration beyond a certain level causes protein denaturation resulting in decreased oil recovery (Tan *et al.*, 2016; Dutta *et al.*, 2015; Sharma and Gupta 2004).

#### 3.2.2. Effect of Sesame slurry/*t*-butanol ratio

The effect of different slurry/*t*-butanol (v/v) ratios (1:0.5, 1:1, 1:1.5, 1:2 and 1:3) of sesame on the extraction of oil by TPP is shown in Figure 2b. It can be observed that a lower volume of *t*-butanol results in a saturation effect, which is an insufficient availability of solvent. This results in insufficient solubilization of the oil present in the seed powder, leading to lower oil recovery. A significant increase in oil recovery was observed with an increase in the volume of *t*-butanol from 1:0.5 to 1:1, which can be attributed to solubilized oil. A maximum recovery of 76.42% (w/w) was achieved with a slurry/*t*-butanol ratio 1:1. With a further increase in the ratio there was not much variation observed in oil recovery. Moreover, the use of *t*-butanol with high volume is uneconomical. The use of a standardized quantity solvent will avoid the saturation effect, and result in the efficient use of solvent and resources (Tavanandi *et al.*, 2018; Tan *et al.*, 2016; Dutta *et al.*, 2015). Accordingly, a sesame slurry/*t*-butanol ratio of 1:1 was intended as the most suitable and maintained constant for the standardization of the most suitable system pH.

#### 3.2.3. Effect of pH concentration on oil recovery

In order to arrive at the most suitable system pH, TPP experiments were performed at salt concentrations of 40% (standardized in section 3.2.1) and *t*-butanol/slurry ratio (1:1) (standardized in previous section), at different pH (range of 3.0 - 9.0). The maximum oil recovery of 78.24% (w/w) was obtained at pH 5.0 and 40% (w/v) ammonium sulphate concentration (Figure 2c). As mentioned earlier, the increase in protein precipitation is an indication of better rupture of the cell wall. According to Tzen *et al.*, (1993), the Isoelectric Point (pI) of proteins from oil bodies varies between ~5.7 - 6.6. At optimum pH (5.0), which is a lower pH than pI, proteins become positively charged and sulphate ions efficiently bind and precipitate them (Vidhate and Singhal, 2013), in turn, producing higher oil recovery. Similar observations were made by other researchers (Dutta *et al.*,

2015). At pH above pI (above 7.0), the oil recovery was found to decrease, with the least amount of oil recovered of 72.42% at pH 9.0.

The best conditions for oil extraction by TPP can be summarized as 40% concentration of ammonium sulphate, 1:1 slurry/*t*-butanol ratio at system pH 5.0.

### 3.3. Enzyme-assisted three-phase partitioning

It was observed that oil recovery in the case of TPP was much lower (~78%) than solvent extraction, which indicated the probability of further improvement for the recovery of oil. One of the eco-friendly approaches is subjecting the sesame seed powder to cell disruption prior to TPP by applying enzymes. Different process parameters that affect EATPP, such as cell disruption parameters, different enzymes and pH) were standardized to effectively hydrolyze a complex seed coat followed by extraction by TPP (at already standardized conditions) leading to enhanced recovery of oil from sesame seeds. This cell disruption step when integrated with TPP resulted in a new and different process called EATPP. The present work integrated disruption and extraction processes which can be considered intra-integration (multiple unit operations in single step)

It is reported that, "*Sesamum indicum*" sesame seed coat contains 42% pectin and other dietary fiber (Elleuch *et al.*, 2012), 62% carbohydrates and 16% proteins (Mbaebie *et al.*, 2010). Based on the composition, three different enzymes (commercial) were selected namely pectinase (hydrolysis of pectin),  $\alpha$ -amylase (hydrolysis of the  $\alpha$ -1,4 glycosidic bonds): amylo glucosidase (hydrolysis of the  $\alpha$ -D-(1-4),  $\alpha$ -D-(1-6) and  $\alpha$ -D-(1-3) glucosidic bonds (1:1) and protease (hydrolysis of the peptide bonds) which are active against the substrates pectin, carbohydrates and proteins, respectively. The Sesame seed powder was treated with each of these enzymes (pectinase, pH 4.0,  $\alpha$ -amylase: amylo glucosidase, pH 6.8 and protease, pH 6.8) prior to the addition of phase-forming components of TPP. The condition already standardized earlier for TPP (40% concentration of ammonium sulphate and 1:1 slurry/*t*-butanol ratio at system pH 5.0) was used. It can be seen from Figure 2d that of all the enzymes studied, pectinase resulted in the highest oil recovery of 86.12%. The oil recovery (86.12%) achieved by the EATPP of sesame seed was observed to be significantly higher ( $p < 0.05$ ) than that of the control (TPP, 78.24%). The present results are in line with those previously reported for enzyme-assisted extraction of oil from canola (*Brassica napus* L.) by Latif and team where the enzyme-extracted oil yield (22.2–26.0%) was found to be significantly higher than that of the control (without enzyme) (16.5%) (Latif *et al.*, 2008). The higher oil yield achieved in EATPP compared with the TPP can be attributed to the better solubilization of the sesame seed

coat/cell body wall that surrounds the lipid bodies, resulting in the liberation of a higher content of oil (Tzen and Huang 1992; Latif *et al.*, 2007; Latif and Anwar, 2009). Such trends were also observed by Soto *et al.*, (2004), who reported a considerable increase in the extraction yield of borage oil due to enzyme-aided cold pressing. Similarly, during the enzymatic-assisted extraction of sesame seed, groundnut, sunflower, cottonseed, hemp seed and flax seed, an improvement in oil recoveries was recorded by researchers (Singh *et al.*, 1999; Latif *et al.*, 2007; Latif *et al.*, 2008; Latif and Anwar, 2009; Anwar *et al.*, 2013).

In case of EATPP, the amount of oil recovered was relatively higher in pectinase-treated seeds, while the sample that was extracted after treatment with the protease enzyme (86.12%) yielded the least amount of oil (80.54%). The enhanced extraction yield in the case of pectinase-assisted TPP (best EATPP method) can be linked to the functional compatibility of this enzyme with the structural composition of the sesame seed coat comprising relatively higher amounts of pectin (42%). It appears that the pectinase efficiently swelled and hydrolyzed the pectin cementation of sesame seeds, enhancing both the oil availability and extractability (Huang, 1994; Tzen and Huang 1992). The cocktail formulation ( $\alpha$ -amylase: amylo glucosidase, 1:1 ratio) was observed not to result in higher amounts (83.42%) of oil recovery. This is a clear indication of the lower role of carbohydrates in the formation of the seed coat. On a contrary, in a research work published by Mushtaq *et al.*, (2015) the cocktail of enzyme (a mixture of cellulase, pectinase and protease; 50:25:25) at ~4% concentration resulted in the highest yield (~30 g/100g), which can be attributed to the presence of pectin and proteins as main building blocks in the seed coat. Even Rosenthal *et al.*, (1996) observed similar contrary results. The enzyme-assisted extraction (EAE) of oils from various natural raw materials has been reported (Latif and Anwar, 2011; Latif and Anwar, 2009; Latif *et al.*, 2007; Sant'Anna *et al.*, 2003; Sharma *et al.*, 2001b; Singh *et al.*, 1999; Che Man *et al.*, 1996). The methods followed in these reports are quite similar to the method followed in the present work except for the matter that EATPP results in relatively higher yield because of the presence of organic solvent in this method compared to enzyme-assisted extraction (EAE).

In the present work, the oil recovery values are in the decreasing order of EATPP by pectinase (86.12%) > EATPP by mixture of  $\alpha$ -amylase: amylo glucosidase (83.42%) > EATPP by protease (80.54%) > TPP (78.24%) (Figure 2d). Accordingly, EATPP by pectinase was concluded as the best and the oil recovered from this method was used for comparison (in terms of oil recovery and quality) with SE and EATPP. It can be seen from Figure 2d that the EATPP by pectinase could achieve better

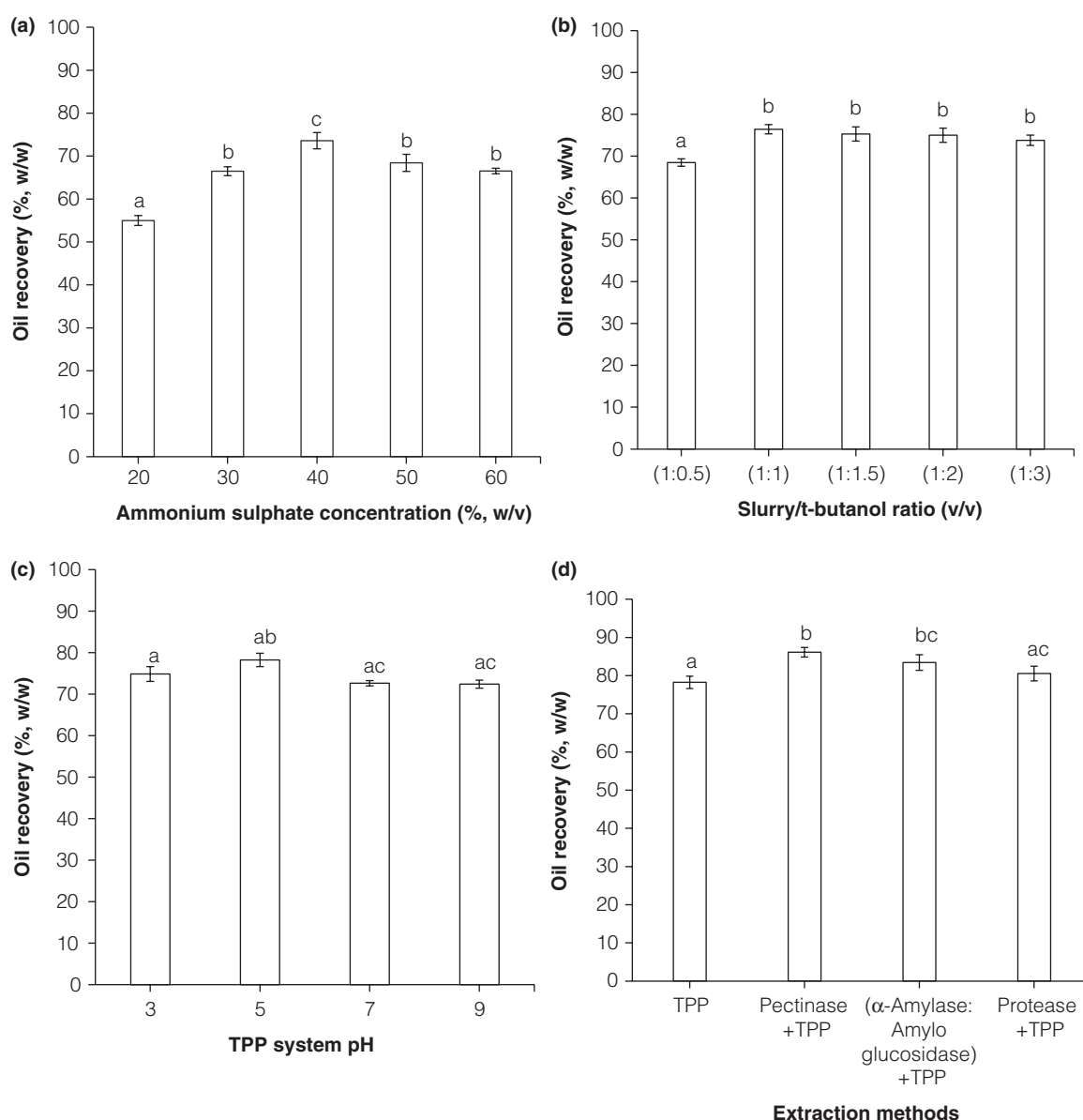


FIGURE 2. Effect of (a) ammonium sulphate concentration (b) sesame slurry/*t*-butanol ratio (c) TPP system pH (d) different extraction methods, on oil recovery. Results are reported as mean values  $\pm$  standard deviation (SD) of triplicate ( $n = 3$ ). Mean values in the same row followed by the different superscripts are significantly different at  $p < 0.05$  (according to one-way ANOVA and Tukey's multiple comparison test).

extraction yield after solvent extraction and the order of oil recovery is as follows: SE (100%) > EATPP (86.12%) > TPP (78%), which indicated that oil recovery is significantly boosted by the enzyme pre-treatment in comparison to TPP (by  $\sim 8\%$ ) alone (un-treated seeds) and close to that achieved by SE. Keeping the higher yield (in line with that of solvent extraction) and all the advantages in mind, EATPP can be considered as the best method. The best condition for oil extraction by EATPP can be summarized as 5% (by seed weight, w/w) pectinase enzyme concentration, pH 4, one hour incubation time followed by TPP at 40%

concentration of ammonium sulphate, 1:1 slurry/*t*-butanol ratio at system pH 5.0.

The enzyme-assisted method, a relatively greener method compared to other conventional methods, was used in the present work. The main advantage of EATPP (and TPP) is that the solvent used is *t*-butanol is preferred over hexane (conventionally used in solvent extraction) since it is considered a much safer, non-toxic solvent, besides being relatively more economical on a large scale. Hence the process prescribed in the present work can be considered relatively greener compared to other conventional methods. This fulfils the overall aim of

TABLE 1. Physico-chemical characteristics of solvent, three-phase partitioning and enzyme-assisted three-phase partitioning extracted sesame seed oil

Parameters	SE	TPP	EATPP (Pectinase)
Free Fatty Acids (% oleic acid)	0.58±0.02 <sup>a</sup>	0.48±0.02 <sup>b</sup>	0.47±0.01 <sup>b</sup>
Peroxide value (meq O <sub>2</sub> /kg of oil)	1.7±0.1 <sup>a</sup>	1.1±0.1 <sup>b</sup>	1.4±0.2 <sup>ab</sup>
Color (1inch cell, 5R+Y Lovibond units)			
Red	1.40±0.02 <sup>a</sup>	1.20±0.03 <sup>b</sup>	1.20±0.02 <sup>b</sup>
Yellow	22.31±0.50 <sup>a</sup>	19.80±0.41 <sup>b</sup>	19.15±0.40 <sup>b</sup>
Saponification Value (mg KOH/g of oil)	176±1.8 <sup>a</sup>	168±1.1 <sup>b</sup>	170±2.0 <sup>b</sup>
Iodine Value (gI <sub>2</sub> /100g of oil)	107±2 <sup>a</sup>	109±1.7 <sup>a</sup>	104±2.6 <sup>a</sup>
<b>Fatty acid profile</b>			
Palmitic acid (C <sub>16:0</sub> )	10.74±0.10 <sup>a</sup>	9.85±0.14 <sup>b</sup>	11.40±0.25 <sup>c</sup>
Stearic acid (C <sub>18:0</sub> )	5.80±0.10 <sup>a</sup>	4.98±0.23 <sup>b</sup>	5.67±0.16 <sup>a</sup>
Oleic acid (C <sub>18:1</sub> )	40.32±0.68 <sup>a</sup>	39.86±0.34 <sup>ab</sup>	38.94±0.26 <sup>b</sup>
Linoleic acid (C <sub>18:2</sub> )	42.51±0.75 <sup>a</sup>	43.81±0.40 <sup>a</sup>	43.28±0.39 <sup>a</sup>
Linolenic acid (C <sub>18:3</sub> )	0.18±0.01 <sup>a</sup>	0.17±0.01 <sup>a</sup>	0.18±0.01 <sup>a</sup>
Arachidic acid (C <sub>20:0</sub> )	0.42±0.03 <sup>a</sup>	0.35±0.06 <sup>a</sup>	0.41±0.05 <sup>a</sup>
SFA	16.96±0.20 <sup>a</sup>	15.18±0.1 <sup>b</sup>	17.48±0.30 <sup>a</sup>
MUFA	40.32±0.68 <sup>a</sup>	39.86±0.34 <sup>ab</sup>	38.94±0.26 <sup>b</sup>
PUFA	42.69±0.70 <sup>a</sup>	43.98±0.40 <sup>a</sup>	43.46±0.40 <sup>a</sup>

Where SE-Solvent extraction; TPP- Three-Phase Partitioning; EATPP- Enzyme-Assisted Three-Phase Partitioning; SFA- Saturated Fatty Acids; MUFA-Mono Unsaturated Fatty Acids; PUFA-Poly Unsaturated Fatty Acids.

Values are reported as means ± standard deviation (SD) of extracted sesame seed oils analyzed individually in triplicate (n=3). Significant mean differences are indicated by different letters (a, b, c). Mean values in the same row followed by the different superscripts are significantly different at  $p < 0.05$  (according to one-way ANOVA and Tukey's multiple comparison test).

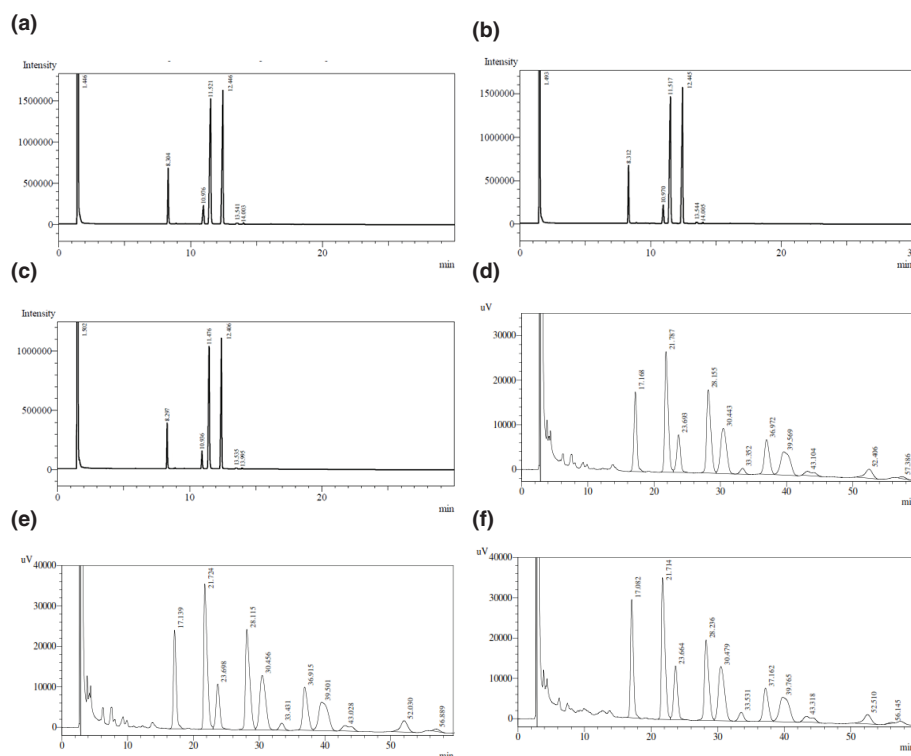


FIGURE 3. GC (a-c) and HPLC (d-f) chromatograms of sesame oils recovered by different methods.



the work, which was coming up with a method that can achieve a similar oil recovery to that of solvent extraction besides being eco-friendly. The advantage of this method is that it does not involve a centrifugation step to separate the cell debris (containing oil) and supernatant (containing protein). The phase components are added directly to the incubated slurry, thereby reducing a unit operation of centrifugation (advantage of integration). Enzymes are very specific with respect to substrate and hence react selectively even at large scale in addition to providing gentle conditions during disruption.

TABLE 2. Triacylglycerol molecular species of solvent based, three-phase partitioning (TPP) and enzyme-assisted three-phase partitioning extracted oil from sesame seeds

Triglyceride molecular species (TG)	(Area %)		
	SE	TPP	EATPP
LLL	11.10±0.4 <sup>a</sup>	10.42±0.3 <sup>ab</sup>	10.15±0.2 <sup>b</sup>
OLL	19.90±0.3 <sup>a</sup>	19.38±0.5 <sup>a</sup>	19.5±0.3 <sup>a</sup>
PLL	6.83±0.1 <sup>a</sup>	7.10±0.2 <sup>a</sup>	6.55±0.4 <sup>a</sup>
OLO	17.90±0.4 <sup>a</sup>	17.90±0.5 <sup>a</sup>	18.6±0.3 <sup>a</sup>
PLO	13.50±0.3 <sup>a</sup>	13.57±0.4 <sup>a</sup>	13.7±0.3 <sup>a</sup>
PLP	1.30±0.3 <sup>a</sup>	1.15±0.1 <sup>a</sup>	1.32±0.2 <sup>a</sup>
OOO	9.70±0.3 <sup>a</sup>	10.11±0.5 <sup>a</sup>	9.5±0.4 <sup>a</sup>
OLS/POO	11.80±0.2 <sup>a</sup>	12.36±0.5 <sup>a</sup>	12.42±0.4 <sup>a</sup>
POP	2.47±0.1 <sup>a</sup>	2.40±0.3 <sup>a</sup>	2.48±0.3 <sup>a</sup>
SOO	3.60±0.1 <sup>a</sup>	3.80±0.2 <sup>a</sup>	4.01±0.2 <sup>a</sup>
POS	1.59±0.2 <sup>a</sup>	1.60±0.3 <sup>a</sup>	1.37±0.03 <sup>a</sup>
UUU	58.61±0.6 <sup>a</sup>	57.82±1.6 <sup>a</sup>	57.75±1.2 <sup>a</sup>
SUU	35.79±0.4 <sup>a</sup>	36.83±0.3 <sup>b</sup>	36.68±0.1 <sup>b</sup>
SSU	5.34±0.5 <sup>a</sup>	5.16±0.8 <sup>a</sup>	5.17±0.04 <sup>a</sup>
SSS	-	-	-

Where P, palmitic; S, stearic; O, oleic; L, linoleic, trisaturated (SSS), monounsaturated (SSU), diunsaturated (SUU) and triunsaturated (UUU), where SE-Solvent extraction; TPP- Three-Phase Partitioning; EATPP- Enzyme-Assisted Three-Phase Partitioning. Values are reported as means ± standard deviation (SD) of extracted sesame seed oils analyzed individually in triplicate (n=3). Significant mean differences are indicated by different letters (a, b, c). Mean values in the same row followed by the different superscripts are significantly different at  $p < 0.05$  (according to one-way ANOVA and Tukey's multiple comparison test).

However, it suffers from the drawback of its limited availability and the high cost of purified enzymes hinders application on a large scale. These problems can be overcome by enzyme immobilization (Crapisi *et al.*, 1993), which reduces the operational cost by lowering the enzyme requirement and loss. Further, employing different size reduction methods in combination with the enzyme-assisted method will result in higher yields and reduce the enzyme concentration required for pre-treatment.

The EATPP method is known not only to achieve higher oil recovery, but also to yield better quality oil. Hence, the quality of the oil obtained by SE, TPP and EATPP are analyzed for their acceptability and the results are discussed in detail in the following sections.

### 3.4. Comparison of physico-chemical characteristics of oil

The physico-chemical properties of the oil extracted using SE, TPP and EATPP were analyzed. The FFA content, PV and SV in both TPP and EATPP were found to be significantly lower than the oil extracted using SE (Table 1). Lower values of FFA, PV and SV indicate better quality of oil. The better results can be attributed to the mild biocompatible environment provided by the EATPP and TPP systems. The fatty acid composition of the sesame oil recovered using SE, TPP and EATPP is presented in Table 1 and Figure 3 a-c. There was no significant difference observed ( $p > 0.05$ ) in the fatty acid compositions of the oil extracted from solvent (40.32 and 42.69% for mono and polyunsaturated fatty acids, respectively), TPP (39.86 and 43.98 % mono and polyunsaturated fatty acids, respectively) and EATPP (38.94 and 43.46% mono and polyunsaturated fatty acids, respectively). Similar results were reported (Latif and Anwar, 2011; Reena *et al.*, 2009) for the sesame oil recovered from the solvent and enzyme-assisted processes. A color analysis of these samples revealed that lower color values were observed in the oil extracted by TPP and EATPP than the solvent (hexane) extracted oil ( $p > 0.05$ ), indicating higher consumer acceptability.

TABLE 3. Melting behavior of oil extracted using solvent extraction (SE), three-phase partitioning (TPP) and enzyme-assisted three-phase partitioning (EATPP) from sesame seeds

Extraction method	Peak			
	Onset (°C)	Peak (°C)	End set (°C)	(J/g)
Solvent (Hexane)	-19.1±0.3 <sup>a</sup>	-6.40±0.2 <sup>a</sup>	-0.33±0.02 <sup>a</sup>	6.144±0.1 <sup>a</sup>
Three-phase partitioning	-23.6±0.4 <sup>b</sup>	-7.36±0.1 <sup>b</sup>	-0.56±0.03 <sup>b</sup>	7.78±0.1 <sup>b</sup>
Enzyme-assisted three-phase partitioning	-22.31±0.2 <sup>c</sup>	-7.98±0.2 <sup>c</sup>	-0.20±0.03 <sup>c</sup>	7.57±0.1 <sup>b</sup>

Values are reported as means ± standard deviation (SD) of extracted sesame seed oils analyzed individually in triplicate (n=3). Significant mean differences are indicated by different letters (a, b, c). Mean values in the same column followed by the different superscripts are significantly different at  $p < 0.05$  (according to one-way ANOVA and Tukey's multiple comparison test).

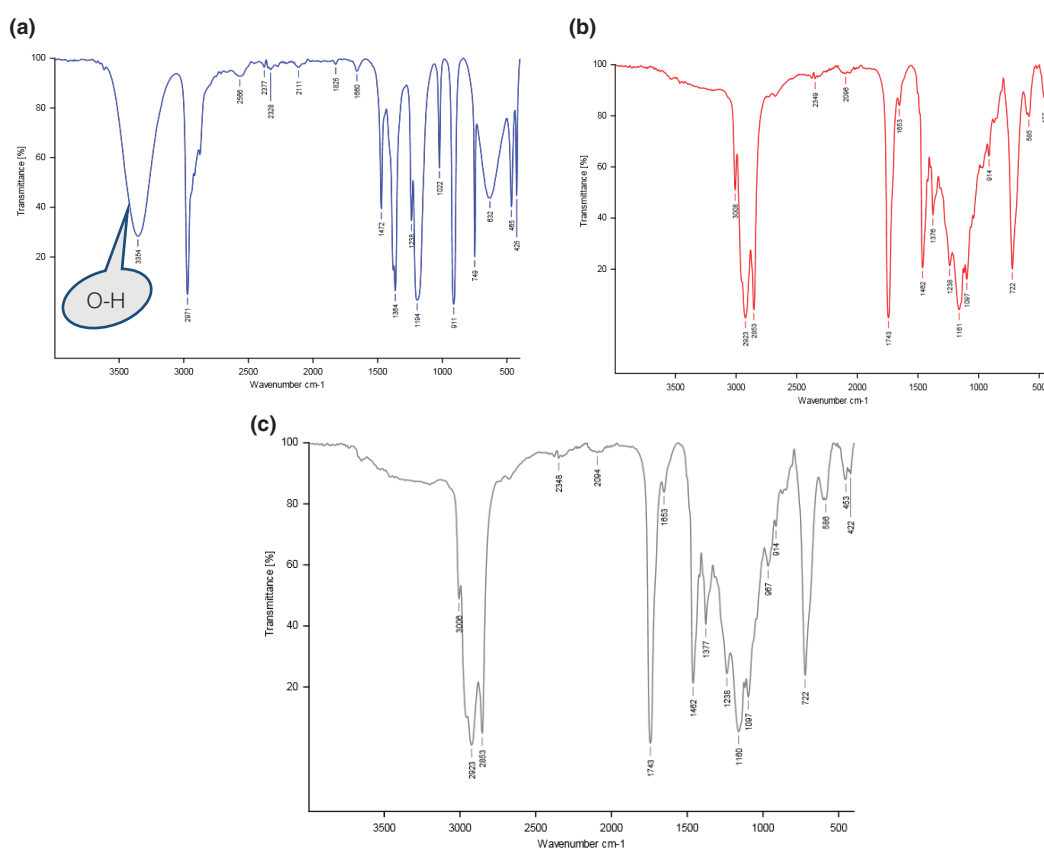


FIGURE 4. FTIR spectrum of (a) *t*-butanol, (b) three-phase partitioning extracted sesame oil and (c) enzyme-assisted three-phase partitioning extracted Sesame oil.

### 3.5. Triacylglycerol molecular species

The triacylglycerol structure determines the thermal and physico-chemical characteristics of oil, which in turn influences its oxidative stabilities and thermal properties (including heat transfer). The triacylglycerol molecular species of the oil obtained from SE, TPP and EATPP are shown in Table 2 and Figures 3 d-f. Eleven different triacylglycerol molecules were identified in the sesame oil samples extracted from these methods, which were similar to the literature reports (Reena *et al.*, 2009). About 93% of the triacylglycerols in the sesame oil extracted by these methods (Table 2) contained di- and tri-unsaturated fatty acids, exhibiting a melting point below  $27 \pm 2^\circ\text{C}$ , resulting in the retention of a liquid physical state even at ambient temperature ( $27 \pm 2^\circ\text{C}$ ).

### 3.6. Melting point profile

The melting profiles of the sesame oil extracted by SE, TPP and EATPP are shown in Table 3. The oil obtained from solvent extraction showed an endothermic peak at the temperature range of  $-19.1$  to  $-0.33^\circ\text{C}$ . The low-melting peaks of the TPP and

EATPP were observed to be in the range of  $-23.6$  to  $-0.56^\circ\text{C}$  and  $-22.31$  to  $-0.20^\circ\text{C}$  (Table 3), respectively.

A shift in peak melting point of the triacylglycerols towards a lower temperature range from  $-6.40 \pm 0.2^\circ\text{C}$  (solvent extracted oil) to  $-7.36 \pm 0.1^\circ\text{C}$  in the oil extracted from EATPP, which further decreased to  $-7.98 \pm 0.2^\circ\text{C}$  in the sesame oil extracted using TPP was observed. However, the enthalpy ( $6.144$  J/g) of the oil from SE was found to decrease by 19-21% when compared to those of TPP ( $7.78$  J/g) and EATPP ( $7.57$  J/g) (Table 3). This may be due to the decrease in di-saturated triacylglycerols in the oil extracted from SE as compared to TPP and EATPP.

### 3.7. FTIR analysis

The results of the FTIR spectroscopy of the samples obtained by *t*-butanol (solvent), TPP and EATPP are presented in Figures 4a-c. In the IR spectra of the oil extracted using TPP and EATPP, the characteristic hydroxyl peak of *t*-butanol at  $3300$ - $3500$   $\text{cm}^{-1}$  was absent, confirming that the samples are devoid of *t*-butanol. These results are in agreement with those reported by Shah *et al.*, 2004, where *t*-butanol was found to evaporate from oil samples at  $50^\circ\text{C}$ .

The physico-chemical and thermal analysis of the sesame oil obtained from SE, TPP and EATPP clearly indicated that the oil obtained from EATPP was superior to the oil obtained from SE and TPP in all these aspects in addition to resulting in higher oil recovery. The added advantage is that the best method is eco-friendly.

#### 4. CONCLUSIONS

In the present work, newer methods such as TPP and EATPP were attempted as an alternative to SE for the recovery of sesame (*Sesamum indicum* L.) seed oil. In this study, it was found that the optimum conditions for maximum oil recovery by three-phase partitioning (TPP) (78.24%) were an ammonium sulphate concentration of 40% (w/v), slurry/*t*-butanol ratio 1:1 (v/v) and system pH 5.0 of different enzymes employed during EATPP, pectinase (5% by seed weight) and TPP at standardized conditions resulted in a higher oil recovery of (86.12%). The better oil quality observed in the case of EATPP (oxidative stability parameters) can be attributed to the mild conditions employed during the process. Further investigation is required to make this simple and greener extraction process economically viable by the immobilization of the enzymes used in EATPP. This process can be used by the edible oil manufacturing industries to achieve good quality oil. The EATPP extraction process for sesame oil has the potential of being an alternative method to SE besides being environmentally friendly.

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