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Extraction and partial purification of a novel enzyme from oil seed cakes of Sunflower and assessing its use for milk clotting activity

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Abstract
Global increase in cheese production with decreased supply of calf rennet is leading to search for coagulant alternatives from available resources such as plant extracts. Therefore, the main objective of this study was to assess the suitability of enzyme extracted from oilseed cakes of Sunflower (*Helianthus annuus*) that can be used as a suitable milk clotting substitute. After extraction, the enzyme was partially purified and characterized. The samples from oilseed cakes of sunflower were powdered and extracts were prepared using 5% NaCl as buffer. The extracts were filtered and fractionated with ammonium sulphate (30-60%). This fraction was assumed to contain the Milk Clotting Enzyme (MCE) which was tested for its Milk Clotting Activity (MCA) and was found to be suitable for milk coagulation. The results revealed that concentrations of 5 ml of Sunflower Enzyme Extract (SFEE) per liter of milk at a pH- 4.5-5.0 and temperature- 45-55 °C were found optimum to coagulate milk. MCA of the SFEE was compared to that of Microbial Rennet (MR). MCA of SFEE indicated its potential as suitable rennet substitute in dairy industry.

Keywords: Ammonium sulphate fractionation, milk-clotting activity, sunflower oilseed cakes, rennet substitutes

1. Introduction
Coagulating enzymes have been used in cheesemaking from thousands of years and they seem to be the oldest known application of enzymes (Harboe *et al*., 2010) [10]. Calf rennet, the conventional milk clotting enzyme obtained from the fourth stomach of suckling calves, which consists of chymosin (EC 3.4.23.4) as the major component and another proteolytic enzyme, pepsin (EC 3.4.23.1).

In general, milk coagulation using calf rennet is the most used procedure. However, the worldwide increase in cheese production, coupled with reduced supply and increasing prices of calf rennet and calf diseases like bovine spongiform encephalopathy (BSE) has led to search for alternative milk-clotting enzymes, as appropriate rennet substitutes (Anusha *et al*., 2014; Shah *et al*., 2013) [2, 18]. Apart from this, some religious factors (Islam and Judaism) and others related to vegetarianism of some consumers have greatly limited their use (Shah *et al*., 2013) [18].

Several milk-clotting enzymes of microbial origin are in use in cheese industry, such as aspartic proteases (APs) obtained from *Rhizomucor miehei*, *Rhizomucor pusillus and Cryphonectria parasitica* (Sumantha *et al*., 2006) [19]. Microbial rennet produced by genetically engineered bacteria has proven suitable substitutes for animal rennet, but increasing attention has been directed toward natural rennet extracted from plants (Ahmed *et al.*, 2009) [1]. The consumer concerns regarding genetically engineered foods (e.g., Germany, Netherlands and France forbid the use of recombinant calf rennet) have led to a growing interest in vegetable coagulants (Egito *et al.*, 2007) [7] such as Cardoon (*Cynara cardunculus*) (Louro Martins *et al.*, 1996) [14] and Aak (*Calotropis procera*) (Sanni *et al*., 1999) [17] etc.

Due to this reason, much research interest has been aroused towards discovering new milk clotting enzymes from vegetable or plant sources which can satisfactorily replace rennet. Most of plant rennets produce cheeses with bitter flavours due to excessive proteolytic activity which limits their industrial use (Egito *et al*., 2007) [7]. Thus, the search for a rennet substitute from plant sources having less general proteolytic activity is highly needed to overcome the above mentioned problem. Oilseed cakes/oil meals are by-products obtained after oil

extraction from the seeds. India holds a significant share in world oil seed production such as rapeseed, groundnut, castor seed, sesame, linseed, soyabean, and sunflower seeds (DAC and FW, 2016) [6]. Similar to chymosin, exhibition of proteolytic activity by the seed extract of *Helianthus annuus* towards k-casein, α-casein and β-casein has been identified (Egito *et al.*, 2007) [7]. Oilseed cakes are rich in protein contents and can be utilized through development of new products and fortification of products (Sunil *et al.*, 2015) [20]. Despite the widespread uses of sunflower oilseed cakes, its use as a source of milk clotting enzymes is not reported yet.

Therefore, the enzymes extracted from sunflower oil seed cakes may also prove a potential milk coagulant to fulfill the demand of cheese industry.

In the current study, the Enzyme Extracts (EEs) from sunflower oilseed cakes have been screened and evaluated for their Milk Clotting Activity (MCA) using skimmed milk as substrate.

2. Material and Methods

2.1 Materials

Sunflower (*Helianthus annuus*) oilseed cakes were procured from 'Pari Animal Nutrition' Khanna, Ludhiana'. Dry Skim Milk Powder (SMP) (Sterling Agro Industries Ltd) was procured from local market, Hisar. Microbial Rennet was procured from Urban Platter (Madmillie, Microbial vegetarian rennet tablets). All the chemicals were procured from reputed firms like SRL, Qualigens, CDH, Hi-Media, Sigma-Aldrich etc. were procured through local dealers of reputed companies. Vivaspin® 20 centrifugal concentrators, Membrane 30,000 MWCO (Sartorius) (for desalting and concentration of proteins) were procured from Sigma-Aldrich.

2.2 Methods

2.2.1 Extraction of crude milk clotting enzyme extract (MCEE)

50 g of sunflower oilseed cake was crushed and mixed with 500 ml of 5% NaCl at 9 °C with continuous shaking over a period of 12 hours and then the samples were filtered to obtain crude extracts. The resulted extract was further centrifuged at 3000 rpm for 15 minutes, and the supernatant was collected, dialyzed at 4 °C and then used as the crude enzyme preparation (El-Sayed *et al*., 2013 with slight modification) [9].

2.2.2 Partial purification of the Crude MCEE

Proteins were precipitated from the crude extracts by using ammonium sulphate at 30% saturation, and the mixture was kept at 4 °C for 45 min before centrifugation (10,000 rpm at 4°C for 10 min). The pellets were discarded, and ammonium sulfate was added to the supernatants to reach 60% saturation in both the cases. After 45 min of incubation at 4 °C, the mixtures were again centrifuged (10,000 rpm at 4 °C for 10 min). The pellets were dissolved in 7.5 ml of pure water and then dialyzed at 4 °C to remove salts (Egito *et al*., 2007 with slight modification) [7].

2.2.3 Concentration of partially purified MCEE

Partially purified enzyme extracts were concentrated using Vivaspin® 20 centrifugal concentrators.

2.2.4 Enzyme assay

2.2.4.1 Preparation of Milk-Clotting Substrate (MCS)

Skim milk was used as a substrate for the assay of milk-clotting activity. It was prepared according to the method of Kawai and Mukai (1970) [12] with slight modification.

Twelve gm of skim milk powder was dissolved in 100 ml solution containing 0.1 M acetate buffer, pH 5 and 0.11 gm $CaCl_2$ (0.01M final concentration) and pH was adjusted to pH- 6.0 and used as substrate for assaying milk-clotting activities.

2.2.4.2 Determination of milk-clotting activity (MCA)

The prepared enzyme extract was assayed for its ability to produce milk-clotting activity using the procedure as described by Berridge (1955) [3] with slight modification. The reaction mixture contained 1.0 ml of enzyme solution added to 10 ml of milk-clotting substrate solution already incubated at 37 °C for 15 min. The end point was recorded when discrete particles were discernible. Calculated as follows:

MCA units = 2400/T x S/E

Where

T = Clotting time (in sec).

S = Volume (in ml) of substrate (milk).

E = Volume (in ml) of enzyme or enzyme extract.

2.2.5 Estimation of total protein concentration in Milk Clotting Enzyme extract

The total protein concentration of sunflower enzyme extract (SFEE) was determined by Lowry *et al*. (1951) [15] method using bovine serum albumin (BSA) as a standard.

2.2.6 Characterization of SFEE (Sunflower Enzyme Extract)

The molecular weight of polypeptides in SFEE was determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (Laemmli, 1970) [13].

3 Results and Discussion

3.1 Determination of milk-clotting activity (MCA) of SFEE

Mean values for MCA of partially purified and concentrated enzyme extract from sunflower oilseed cakes have been presented in Table 1. The Milk Clotting Activity (MCA) of control (microbial rennet) was 550.00 units/ml of MCS and was found significantly ($P \leq 0.01$) higher as compared to that of SFEE (210.00 units/ml). This might be owing to the fact that Microbial Rennet (MR) is a purified milk coagulating enzyme whereas the milk coagulating agent used for the present study (plant derived) is the partially purified enzyme, extracted from sunflower oilseed cakes. Egito *et al.* (2007) [7] carried out the studies on MCA found in ammonium sulphate precipitated protein extracts from *Albizia lebbeck* and *Helianthus annuus* seeds and concluded that MCA of protein extracts corresponding to *Albizia lebbeck* and *Helianthus annuus* seeds were 155 and 3.9 units (mg of extract/ ml of MCS), respectively. The work done by Ahmed *et al.* (2009) [1] on characterization of partially purified milk-clotting enzyme from *Solanum dubium* Fresen seeds indicated that the MCA of MCEE was 880 units/ml and those belonging to Mucor rennet and Papain were 551 and 216 units/ml respectively.

Table 1: Milk Clotting Activity (Mean ± SD, n=6) of SFEE and MR

Enzyme/Enzyme Extract	MCA (units/ml)
Sunflower Enzyme Extract (SFEE)	$210.00^{a} \pm 0.55$
Microbial rennet (MR)	$550.00^{b} \pm 1.15$

Means with different superscripts differ significantly ($P \leq 0.01$)

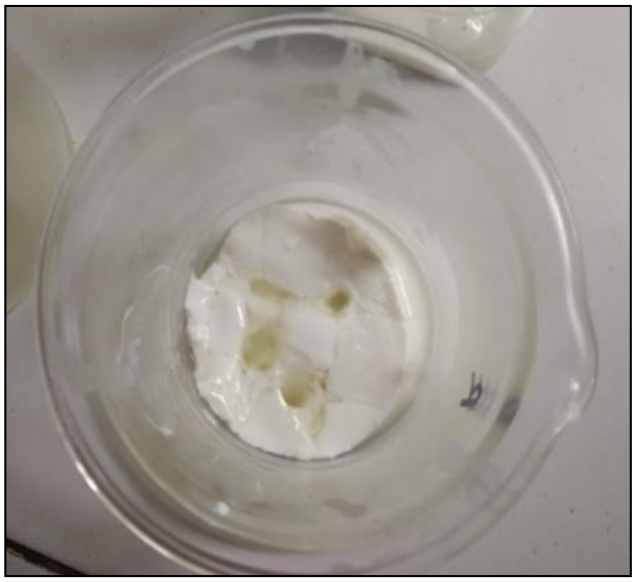

Fig 1.1: Coagulation with Microbial Rennet

Fig 1.2: Coagulation with Sunflower Enzyme Extract

3.2 Estimation of total protein concentration in Sunflower Enzyme Extract (SFEE)

The total protein concentration (mg/ml) of SFEE has been presented in table 2. The total protein concentration (mg/ml) of SFEE (100.00) was significantly higher as compared to that pertaining to MR solution (15.04).

Concentration of purified MR solution recommended for cheesemaking is as low as 15 mg/ml of enzyme solution to coagulate 1 litre of milk (1.5 g/100 litres of milk as described by Jana and Mandal (2011) [11]. It reveals that very low concentration of purified enzyme of microbial rennet is sufficient to coagulate milk to make a good quality cheese. On the other hand, the protein concentration of enzyme extract derived from sunflower oilseed cake (SFEE) was estimated first using Lowry protein assay and then optimum concentration of this extract required to coagulate a given quantity of milk was standardized. In this context, Egito *et al.* (2007) [7] worked upon ammonium sulphate precipitated protein extracts from *Albizia lebbeck* and *Helianthus annuus* seeds to find MCA. The total protein concentrations of these extracts were evaluated as 263.9 and 100.8 mg/ml, respectively.

Table 2: Total protein concentration (Mean ± SD, n=6) of SFEE and MR

Enzyme/Enzyme Extract	Total protein (mg/ml)
Sunflower Enzyme Extract (SFEE)	$100.00^{b} \pm 1.17$
Microbial rennet (MR)	$15.04^{a} \pm 1.01$

Means with different superscripts differ significantly ($P \leq 0.01$)

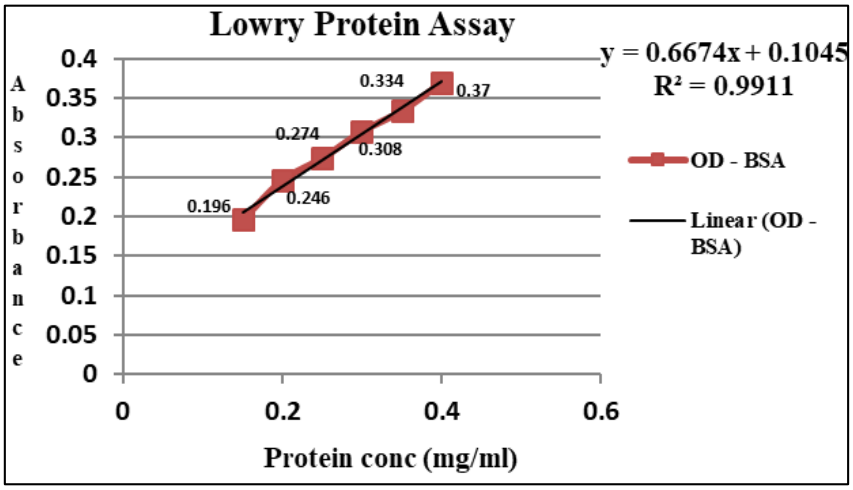

Fig 2: Standard curve (BSA)

3.3 Characterization of SFEE (Sunflower Enzyme Extract)

The electrophoretic behaviour of SFEE and MR (control) on Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS- PAGE) is shown in figure 3. Lane 5 represents MR band pattern and Lane 6 belongs to protein molecular marker. Lanes 1, 2, 3 and 4 represent protein band pattern of SFEE. Lanes 1 and 3 (SFEE) are the lanes representing band pattern

of enzymes extracted 3 months earlier which were kept in frozen conditions whereas lane 2 and lane 4 represent band pattern of fresh enzyme extracts. The figure shows that MR exhibits single protein band at 45 kDa (Lane 5). The figure corresponding to lanes 1-4 reflected that inspite of having protein bands of different molecular weights; the prominent bands in the respective lanes (1-4) represent proteins having molecular weights in the range of 35- 45kDa. Ahmed *et al.* (2009) [1] reported the results of SDS-PAGE of partially purified enzyme from *Solanum dubium* seeds. They applied 35-55% ammonium sulphate fractionation as a first purification step and when SDS-PAGE of this particular partially purified fraction was carried out; they found that this fraction contained multiple bands of polypeptides. However, it was depicted that ammonium sulphate fractionation of crude extract of enzyme led to the salting out of over 86% of the total proteases.

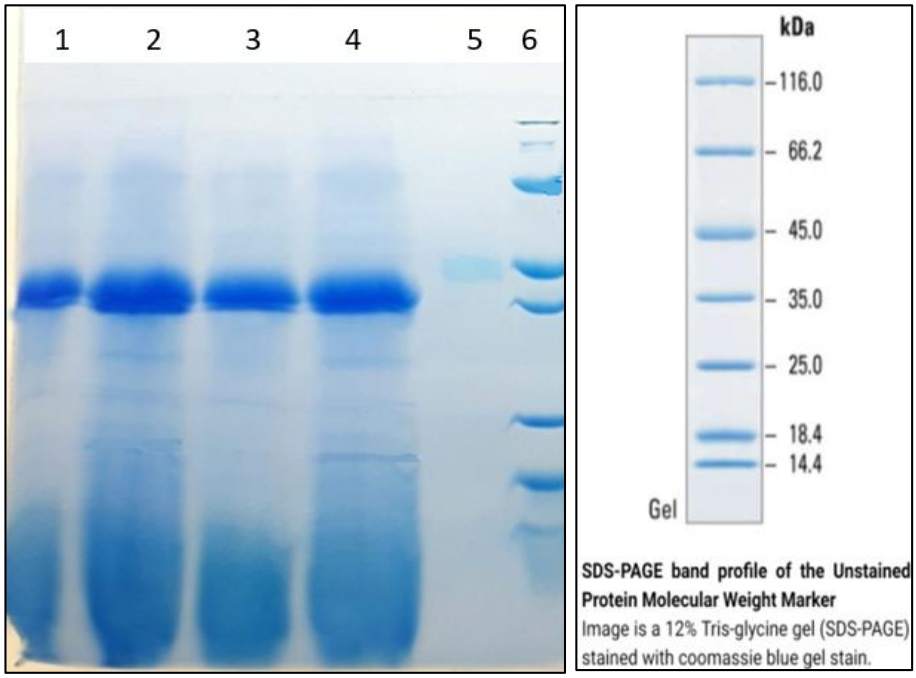

Fig 3: SDS- PAGE pattern of SFEE

3.4 Physicochemical properties of partially purified MCEE

3.4.1 Effect of pH value on the Milk Clotting Activity (MCA) of SFEE

Figure 4 shows the effect of pH on MCA of SFEE as well as of MR. For SFEE, at pH value 6.5 and above, the enzyme extract failed to clot the milk. Maximum MCA was found at pH- 4.5-5.0 for SFEE and pH- 5-6 for MR. It shows that the enzymes responsible are acidic proteases that make full activity at low pH. Results agree with the findings of Elmazar *et al.* (2013) [8] who concluded from their work on *Brassica napus* seed extract for MCA, that the optimum pH value for milk clotting activity was at 4.5 whereas a notable decrease in MCA was progressively evident as the pH of buffer increased toward neutrality and above. Park *et al.* (2000) [16] reported that the purified aspartic protease from *Helianthus annuus*, showed almost negligible milk-clotting activity determined at 35 °C and pH 6.0. Reports for milk clotting enzymes isolated from plants as *Cynara scolymus* L. flower showed a loss of activity of 87% at pH 7 (Chazara *et al*., 2007) [4].

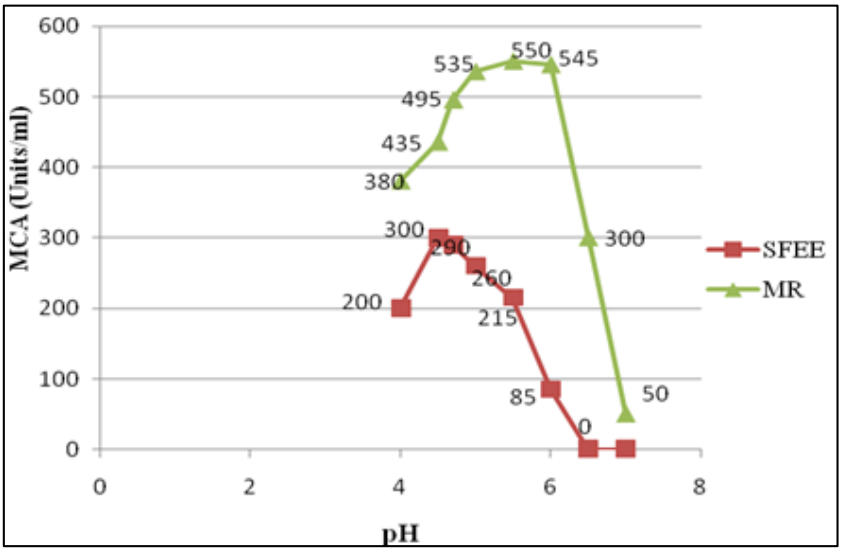

Fig 4: Effect of pH value on MCA of SFEE

3.4.2 Effect of temperature on the Milk Clotting Activity (MCA) of SFEE

Figure 5 shows the effect of temperature on MCA of SFEE and MR. MCA increased progressively with the increase in temperature of reaction mixture (mixture of MCS and enzyme solution) reaching the highest MCA value at 45-55 °C for SFEE and 35-45 °C for MR. At higher temperatures (at 65-70 °C), loss of activity was found in the enzyme solutions. The temperature profile of the enzyme extracts (MCEEs) was agreed with those of El- Sayed *et al.* (2013) who studied the effect of temperature on the MCA of purified MCE obtained from *Brassica napus* seeds. According to them, the MCA increased progressively with the incubation temperature reaching the highest MCA value at 60ºC. At higher incubation temperatures, total loss of MCA was found.

3.4.3 Effect of concentration on the Milk Clotting Activity (MCA) of SFEE

The MCA of the enzyme extracts was determined at different concentrations of enzyme extracts ranging from 0.1-0.7% v/v (ml/100 ml) of reaction mixture. For purified MR, MCA was observed at its already recommended concentration in milk i.e. 0.0015% (1.5g/100 l of milk) (Jana and Mandal, 2011) [11]. It was observed that MCA was increased proportionally with the increase in concentration up to 0.7% v/v of reaction mixture in case of SFEE. However, the MCA of MR (control) at 0.0015% concentration was significantly higher (550 units/ml) than that belonging to the plant extracted enzyme extract (SFEE) at any of taken concentrations. According to Chitipinityol and Crabbe (1998) [5] the milk clotting time decreases with increasing enzyme concentration.

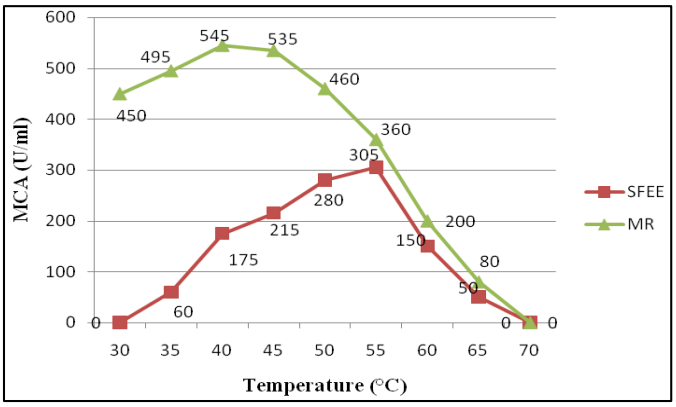

Fig 5: Effect of temperature on the MCA of SFEE

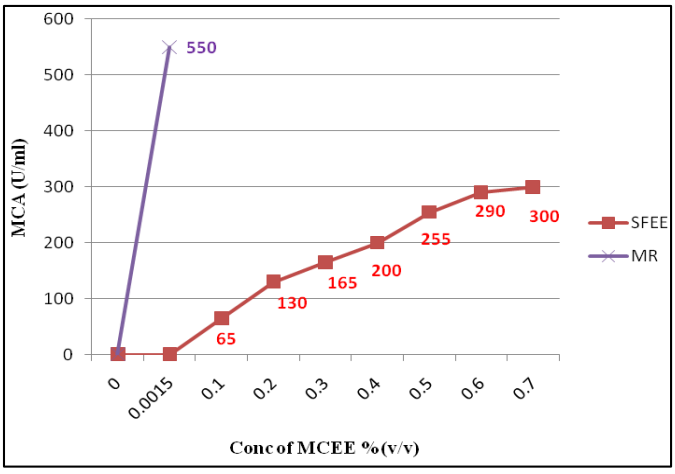

Fig 6: Effect of concentration on the MCA of SFEE

4. Conclusions

To the best of our knowledge, this is the first report of the partial purification of a milk-clotting enzyme from the oilseed cakes of Sunflower (*Helianthus annus*). The simple and cheap procedure for partial purification of the enzyme applied in the current study, along with the availability of the sunflower oilseed cakes, could possibly be used for large-scale production of the enzyme, allowing a broad study of its various aspects and hence probable applications. Additional studies on the complete purification and characterization of this promising enzymes together with the intense evaluation of the quality of cheese curd produced by its action will shed more light into its commercial suitability.

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