

Solid State Fermentation of Orange Peels for Production of Cellulase, Pectinase and Recovery of Orange Oil using Aspergillus Species NCIM 1432.

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Research

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Abstract

The conventional orange peel extraction processes have several drawbacks hence; there is a need to develop green process for extraction of orange oil. In present study, solid state fermentation of fresh orange peels by *Aspergillus species* NCIM -1432 to extract extra-cellular enzymes and orange peel oil are noted and the effect of methods of orange peel sterilisation, importance of selection of the fermentation strain, external nitrogen source, and particle size of the peels are also studied.

The process used here gave 0.95 % w/w yield of orange oil with 96 % w/w limonene content with typical orange colour and flavour. Also, the crude enzymes produced during fermentation showed 155 U/ g and 239 CMC/ g respectively of pectinase and cellulase activity. The molecular weights of the obtained enzymes were found to be 39, 55 and 120 kDa which corresponds to the reported pectinase and cellulase.

Introduction

Enzymes have been utilized in past in bioprocessing for various applications. Whole-cell organisms have been the resources to produce the extracellular as well as intracellular enzymes. Extracellular enzymes are particularly attractive as their recovery is simpler than the intracellular enzymes.

An *in situ* production of enzymes for recovery of oil from the orange peels is very attractive. The methods employ either submerged fermentation (*SmF*) or solid-state fermentation (*SSF*), the latter being more common. Mrudula and Anitharaj, [1] reported production of pectinase enzyme by *Aspergillus niger* using orange peel as the substrate. Mamma et al., [2] reported production of pectinolytic, cellulolytic and xylanolytic enzymes by mesophilic *Aspergillus niger*, *Fusarium oxysporum*, *Nwuraspora crassa*, and *Penicillium decumbens* under *SSF*.

Schuster et al., [3] have reviewed *Aspergillus niger* to produce extracellular enzymes for bio-transformation of vegetable waste to citric acid. The authors have pre-treated peels to remove the orange oil before fermentation of the orange peels as *Aspergillus* or yeast strains quickly grow on limonene-free peels.

It is challenging to store the orange peels as the peels are susceptible to microbial degradation due to their high moisture and carbohydrate content. As against the moisture and carbohydrate content in the peels which supports microbial growth; the limonene content in the orange peels inhibits the biological activity of the microbes. Hence, to overcome the issue related to inhibition of biological activity, Omran et al. [4] removed orange oil before fermentation of peels with *Aspergillus species*. Also, the limonene in orange oil has allergenic properties which makes the peel unsuitable as cattle feed [5].

Rivas et al., [6] and Ahmed et al. [7] have reported production of sugar and pectinase enzyme via *SmF* of orange peel, where the orange peels were converted to liquor or supplemented with Czapeck media. In *SmF*, dry orange peel powder or orange peel hydro-lysate or liquor are added to the aqueous medium or are supplemented with synthetic media (Czapeck) for the production of cellulolytic and pectinolytic enzymes, citric acids, flavonoids, bio-fertilizers, proteins, orange flavour, and fermentable sugars [8- 9].

Suneetha et al., [10] and Wikandari et al., [11] mentioned advantages of *SSF* over *SmF*. The *SSF* provides anchorage for the growing microbial cultures and also prevents bacterial contamination due to low moisture content. There are several reports on production of various chemicals including isothiocyanates from *Brassica* vegetable, corn oil, palm oil, soyabean oil, coconut virgin oil, and citric acid by *SSF* and enzymes [12– 16]

The literature indicates that the *SSF* improves the production of enzymes, antibiotics, organic acids, pesticides, aroma, and bio-fuel. However, there are no reports on orange peel oil extraction by the generation of essential enzymes by fermentation [11, 17-18]. Based on the results obtained by using enzymes for a cellulolytic activity for enhancing orange oil extraction and understanding the use of fungal strains to generate the cellulolytic enzymes, the current work has used *SSF* of orange peels to enable extraction of orange oil.

The fungal hyphae penetrate the polymeric structure of peel, absorb the nutrients from the peels and the produce the enzymes that hydrolyse cell wall and enable the extraction of orange oil.

SSF has been described as the process that takes place in a solid matrix (inert support or support/substrate) in the absence or near absence of free water, but the substrate requires moisture to support the growth and metabolic activity of microorganisms. *SSF* overcomes the resistance of microorganisms (bacterial and fungal cells) to catabolic repression (inhibition of enzyme synthesis) in the presence of substrates, such as glycerol, glucose or other carbon sources in abundance. These aspects of *SSF* mechanism make it an optimum method of choice in the present research.

Materials And Methods

Absolute ethanol (99.99 %) was obtained from Adarsh Scientific Corporation, Mumbai. Limonene standard (99.00 % pure), potato dextrose medium, nutrient agar, nutrient broth, Congo Red, Mandel's salt including ammonium sulfate, urea, calcium chloride, magnesium chloride, ferrous

sulfate, manganese sulfate, zinc chloride and cobalt chloride were obtained from Hi-Media Pvt Ltd., Mumbai.

Fresh orange peels were procured from local fruit juice vendors. Chemicals including citric acid, carbazole, dinitro salicylic acid, glucose trisodium citrate, sodium hydroxide, hydrochloric acid and sulphuric acid of LR grade and solvents such as n-hexane, acetonitrile, methanol of AR Grade were obtained from S.D. Fine Chemicals Ltd., Mumbai. The standard protein markers of 10 to 100 kDa (Kilo Dalton) range was procured from Fisher Scientific and Microfilters (0.2 µm), an ultrafiltration membrane of 100 KDa (Kilo Dalton) from Millipore Pvt. Ltd., while dialysis membrane (24° A) was purchased from Thermo Fisher Scientific

Orange peel particle size reduction

Household mixers equipped with a coarse and a fine blade were used to obtain the orange peels in different particles sizes.

Selection of orange oil extracting Micro-organism

The orange peels were fermented by naturally growing fungal flora (white, green and black). The different colonies grown on orange peels surfaces were streaked on agar plates and then individual colonies were propagated on agar media in glass petri plates to isolate the colonies. Then orange peels were fermented with the isolated colonies which were of different colours (brown, green, black). The colony that enabled production of orange peel oil with characteristic organoleptic properties (orange colour and orange fruit flavour) was selected for further oil extraction by fermentation process [19].

Identification of selected Fungal colony

The selected colony was examined using an Olympus BX- 51 optical microscope. The strain was further confirmed by fungal-specific 28S *rRNA* gene sequencing and Basic Local Alignment Search Tool (BLAST) at National Chemical Laboratory (NCL), Pune.

Optimization of Seed culture medium and Seed Culture Size

Nutrient agar (NA) (0.5 % peptone, 0.3 % Beef extract, 0.5 % sodium chloride, 1% carboxymethylcellulose, 1.5 % agar), Nutrient broth (NB) (0.5 % peptone, 0.3 % Beef extract, 0.5 % sodium chloride, 1% carboxymethylcellulose) and orange peels media were studied for seed culture development. The seed size of 10^1 fungi cm^{-3} to 10^6 fungi cm^{-3} was studied to find an optimum seed culture size. The growth curve was determined based on a dry weight by collecting the fermented seed cultures on a pre-weighed filter paper (*Whatman 1*) and drying them at 60° C for 24 h.

Screening of orange peel sterilisation technique

To enable propagation of single fungal colony on fresh orange peels, three sterilisation techniques were adopted. These include firstly **High pressure steam sterilization method** in autoclave, operated at 121 °C, 15 psi pressure for 15 min. The second is **UV light irradiation treatment** in laminar flow UV-light chamber using a tray covered with transparent plastic cover. Finally, the third one is **Surface Sanitization** by swabbing peel surfaces with 70 % v/v ethanol and the sanitized peels were kept in sterile container.

Optimization of Fermentation parameters

Only fresh and sterile orange peels were used for fermentation in 0.15 m diameter petri plate and were inoculated with the selected fungal strain. The effect of temperatures (8 to 37° C), particle sizes (0.16 mm, 0.2 mm, 1 cm x 1 cm), and concentration of tryptone as an external nitrogen source (1 to 3 % w/w) were investigated on extraction of oil, production of enzymes and fermentation period was investigated.

Monod Kinetics:

Monod Kinetics was determined to understand the specific growth rate and doubling time of the isolated colony in different fermentation medium

The equations used for the determination of 'k' the specific growth rate or the growth constant is

$$\ln\left(\frac{C_f}{C_o}\right) = k(t_f - t_o) \quad (1)$$

$$t\left(\frac{2}{1}\right) = \left(\frac{\ln 2}{k}\right) \quad (2)$$

Where, C_f = Final microbial concentration, C_o = Initial microbial concentration, k = specific growth rate/ rate constant, t_f = final fermentation time, t_o =initial fermentation time

Extraction of fermentation products

The oil and enzyme (s) were extracted from the fermented orange peels by manually compressing the peels, in 50 cm³ of either deionised water or 50 mM citrate buffer solutions of varying pH values.

The residual solid and aqueous phases were separated by filtration followed by centrifugation at 200 rpm for 5 min, and the peel residue was washed thrice with 10 cm³ of buffer solution at optimized pH value of pH 5, to recover the enzyme from the fermented peels.

Purification of enzymes

100 cm³ of the aqueous extract containing crude enzyme was initially filtered through a micro-filter (0.2 µm) to separate the debris. Further fractional precipitation from the filtrate was performed using 20 to 80 % w/w of ammonium sulphate at 4° C, and the final mixture was left for 16 h stirred continuously on magnetic stirrer to allow a complete precipitation of proteins. The suspension was centrifuged, and the solid pellet was dissolved in 10 cm³ of 50 mM citrate buffer (pH 5.5) and dialysed against 50 cm³ of 50 mM sodium citrate buffer (pH 5.5) at 4° C by replacing the buffer three times. After each step, the aqueous phase samples were collected and analysed for enzyme activity.

Enzyme Characterization

All enzyme activities were assayed in duplicate using 50 mM citrate buffer of pH 5 unless noted otherwise. The zone of inhibition study on Mcconkey's agar medium initially enabled to determine the presence of cellulase in the solution. The cellulase concentration was quantified by measuring the reducing sugar concentrations using DNSA method.

Cellulase Activity [20]

A 0.1 cm³ aliquot of appropriately diluted enzyme extract was mixed with 0.4 cm³ of CMC solution (1 % w/v) and was incubated for 30 min at 50 °C. The reaction was stopped by the DNSA reagent followed by heating the mixture to 100 °C. One unit of cellulase activity, one µ mole of glucose released in 30 min from the substrate denoted as CMC cm⁻³.

Pectinase Activity [21]

The pectinase activity was determined by mixing 0.1 cm³ aliquot of appropriately diluted enzyme extract with 0.4 cm³ of pectin solution (1% w/v) and was incubated for 30 min at 50 °C. The reaction was stopped using a potassium iodide solution, and iodimetric titration was performed using 0.2 mM sodium thiosulphate solution and starch as an indicator. One unit of pectinase activity (U cm⁻³) is defined as one µmole of galacturonic acid released from the pectin substrate [22]. The Bradford assay was used to measure the protein content [23].

Enzyme Kinetics

The Michaelis Menton [24] and Lineweaver Burk plots were used to determine the K_m and V_{max} values. The slope of the Lineweaver Burk plot was equal to the K_m/V_{max} and the intercept of the Lineweaver Burk plot on the y-axis is $1/V_{max}$.

Spectro- fluometry

To confirm the extraction of enzymes into the aqueous extract, the fluorescence emission spectrum of the aqueous solution was recorded at room temperature (25 ± 1° C) using a Jasco FP-6500 fluorescence spectro-fluorometer, using the excitation wavelength of 280 nm over the emission wavelength range of 300 to 500 nm [25].

SDS-PAGE

The extracted enzyme was characterized by performing a reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) [26].

Characterization of Oil

Initially, the organoleptic test was used to characterise the extracted oil, the oil was then characterised both quantitatively and qualitatively by gas chromatography-Flame ionisation detector- mass spectrometry (GC-FID-MS) and high-performance liquid chromatography- photodiode array detector- mass spectrometry (HPLC-PDA-MS).

Results And Discussion

Selection of suitable colony for orange oil production by SSF

The oranges peels stored at 8 °C showed the growth of several natural microbial floras on its surface (Fig. 1a). The macro-morphological characteristics (Fig. 1b) and microscopy studies of the flora growing on the peel indicated the fungal flora belonging to *Aspergillus species* (Fig 1c).

The fungal culture absorbs nutrients from the orange peels and secret cell wall degrading enzymes that breaks down the complex structures of cellulose and pectin of the cell wall. This then enables the rupture of the oil glands [27].

The earlier reports of Mrudula and Anitharaj [1], Torrado et al. [1] and Oberoi et al. [28], have reported production of enzymes by *SSF* of orange peels using *Aspergillus niger*. However, these reports have not reported on extracting of the orange peel oil. Hence, we demonstrate the recovery of the orange peel oil from the peel cellular matrix using fermentation.

The manual compression of the fermented peels enabled the physical separation of the oil and aqueous phase from the fermented peels. However, oil had uncharacteristic colour (**Fig. 2**) and odour. Agrawal and Bosco [12], Vu et al. [29], Jaiswal and Ghannam [30] have reported that the fungal strains produce pigments which get solubilised in the oil, making the oil coloured. Thus, the fermentation of orange peels produced oil and the aqueous phase with the colour corresponding to the pigment colour secreted by strain fermenting the peel (**Fig. 2**).

Selection of orange peel sterilization technique

Fungi produce pigments which affect the quality of the oil. Therefore, to obtain orange oil that qualifies in the organoleptic test, it was necessary to obtain contamination free fungal growth medium. Thus, sterilization of peels was important [19]. However, there were some challenges associated with each sterilization method, as mentioned below-

High Pressure steam sterilization

When high pressure steam was used for sterilization, the essential oil from peels escaped out of the autoclave due to the high temperature conditions. The fragrance of orange oil was noticeable in the autoclave room.

Golmohammadi et al. [31], have reported extraction of orange oil using steam explosion at high temperature and pressure in less time compared to hydro-distillation. Yadav et al. [32], reviewed the use of saturated or superheated steam for the extraction of orange peel oil. Unfortunately, in most such cases, without appropriate provision to condense steam, the product also escapes into the atmosphere. The steam-assisted extraction technique requires high-pressure equipment and makes process energy intensive. Kannagi and Elangeshwari [33], also conducted fermentation of the steam sterilized orange peels for cellulase production using *Aspergillus* species, but have not reported extraction of orange oil which might have been escaped from the peels during autoclaving process. Hence, the steam sterilization of orange peels is not a suitable method to obtain fermentation assisted oil from the peels.

UV-light sterilization of orange peels

When the orange peels were subjected to UV light assisted sterilization for 1 to 3 h, the peels got dried. Also, the color of peels was affected. The changes in the orange peels were because of significant loss of moisture from the peels due to heat generated by the UV light and the dry air flow in the laminar flow hood. The UV-light treatment still did not prevent the microbial contaminations which could be because of the limited penetrating ability of UV-light in the solid peel matrix leading to *incomplete* sterilization of peels [34].

Sanitization of peels with 70% ethanol

The surface sanitisation of orange peels using 70 % v/v ethanol was found to be more appropriate as it prevented the contamination, oil evaporation, and the drying of the peels as seen in case of autoclaving and UV-light methods. Gurulingappa et al. [35], have reported the surface sterilisation using 70 % ethanol in tissue culture laboratories as an effective method which is in sync with the findings of the present research.

The manual compression of orange peels, alcohol sterilized and fermented by the isolated white fungal strain, produced characteristic orange oil from the peels as seen in the **Fig 4 (a-e)**

Characterization of isolated fungi

The selected fungal strain showed filamentous growth peculiar to *Aspergillus* strain (**Fig. c**) [36]. The colony producing favourable quality orange oil appeared as white growth when seen with naked eyes (**Fig. 3 b**). The 28 S RNA gene sequencing and BLAST study confirmed the strain as *Aspergillus species* resembling *A. caninus*, *A. inslitus*, *A. chavalieri*, *A. avenaceus*. The isolated species was with the following sequencing report and deposit number *Aspergillus sp./NCIM 1432*.

The isolated *Aspergillus species* possess similar morphological features as that of *Aspergillus niger* [37]. The *Aspergillus species* are generally known to infect food and fruits and cause their spoilage and are rarely regarded as an agent to cause opportunistic infections. These are generally recognised as safe (GRAS) category of fungi by the U.S. Food and Drug Administration (FDA) [38]. The *Aspergillus species* consume organic matters at favourable humidity and temperature conditions and are suitable for industrial fermentation processes [39]. The selected *Aspergillus species/NCIM 1432*, grown on an agar plate showed initial white growth upto 12 h of fermentation period (**Fig. 5 a**), and then, formed black spores (**Fig 5 b**).

Development of seed culture medium

Puri et al. [40], mentioned that it is important to the optimized seed culture medium and size based on which the following three mediums were studied for development of seed culture.

Orange peel as seed culture medium

The *Aspergillus speices/ NCIM-1432* when cultivated on orange peel showed 26 days of lag period hence orange peels as such was not suitable medium for the culture development (Fig. 5 b).

Nutrient Broth as seed culture medium

When the seed culture was developed in nutrient broth, the culture showed the lag phase for 3 days. The seed culture gave an exponential growth on 3rd day and 4th day where the fungi showed a loose filamentous pellet structures with about 25-30 number of pellets per 10 cm³ of nutrient broth (Fig. 5 d). The culture attained the stationary phase on 5th -6th day of fermentation and the growth declined on 7th day onwards. The short exponential growth and rapid completion of stationary and decline phases in case of the *SmF* could be attributed to the agitation during *SmF* process which may break the filamentous fungal strain [41]. The shear forces created by the agitation during the *SmF* cause breaking of the loose filamentous fungal pellets when the eddy size in the fluid is smaller than the cell size [42].

Nutrient Agar as Seed culture medium

The seed culture development on nutrient agar showed just a 6 h of lag phase and gave an exponential growth till 72 h of incubation (Fig. 5). Considering the time required to attain log phase growth of the *Aspergillus species/ NCIM 1432*, the nutrient agar was selected for preparing the seed culture. The solid nutrient agar was appropriate for the growth of *Aspergillus species/ NCIM 1432* when compared to nutrient broth medium.

The nutrient agar contains the nutrients that provide nourishment to the micro-organisms and enable efficient sub-culturing of the microorganism. The addition of agar solidifies nutrient agar, which makes it suitable for the cultivation of micro-organisms and is used for the isolation and preservation of bacterial culture. On the other hand, nutrient broth remains in liquid form at room temperature and are used to maintain the stocks of microorganisms and are used to grow fastidious organisms and to prepare bacterial culture for growth and cultivation for production.

Both the nutrient broth and nutrient agar medium are synthetic media with known composition and a defined amount of carbohydrates, nitrogen, and vitamin sources. The seed culture growth in both the mediums is depicted in Fig. 5 a. The Monod Kinetic plot shows that the specific growth rate was linearly proportional to substrate concentration up to 20g.L⁻¹ for NA medium and 5g.L⁻¹ in case of NB. At the concentration greater than 20 g.L⁻¹ for NA and 5 g.L⁻¹ for NB, a steady state of growth was observed (Fig. 5 b, c). The specific growth rate for the isolated *Aspergillus species* in NA and NB medium was 2.05 x 10⁻² mg.h⁻¹ and 2.53 x 10⁻² mg.h⁻¹ respectively. The doubling time observed for the isolated *Aspergillus species- NCIM 1432* in the NA and NB medium was 3.37.h and 2.74 h respectively.

Gibbs et al. [41], Janet et al.[43] have reported that *SmF* is not suitable for the growth of fungi. Mariano et al. [42], demonstrated that the shear force damages the filamentous fungi during *SmF*, and only the surface of the fungal pellets are metabolically active, while low viability zones exist within the pellets. Further, Basu et al. [44], in their literature review and Janet et al. [43] and Mariano et al [42] in their research studies have demonstrated that solid medium is the most natural and suited medium for fungal growth.

Effect of seed culture size on Fermentation period

The seed culture of *Aspergillus species/ NCIM-1432* grown on nutrient agar medium was collected using different volumes of 0.5 % saline water to obtain different seed sizes from 10 to 10⁶ fungi cm⁻³.

The impregnation of 1 x 1 cm sized orange peels with the seed culture size of 10 fungi cm⁻³ (Batch 1), 10² fungi.cm⁻³(Batch 2) and 10⁴ fungi cm⁻³ (Batch 3), had lag-period of 26, 18 and 7 days of fermentation, respectively, while the seed culture size of 10⁶ fungi.cm⁻³ (Batch 4) on 1 x 1 cm sized orange peels had the lag phase of 15-16 days (Fig. 6).

Thus, the optimum seed culture size was 10⁴ fungi.cm⁻³ which enabled the mat growth of fungi on the peel surface in 8-15 days. With the increased seed culture size of 10⁶ fungi. cm⁻³ the growth rate decreased because of the limited space and nutrients available for propagation of the loaded seed culture. Puri et al., [40], Wu et al. [45], have also projected that optimum seed culture size enhances the efficiency of fermentation.

Effect of orange peel particle size

The impregnation of orange peels of average size 1 cm x 1 cm with the selected *Aspergillus sp./ NCIM-1432* and the seed count 10⁴ fungi cm⁻³ showed the lag phase of 7 days, gave exponential growth from 8th day till 15th day where the fungi covered the surface of orange peels entirely on 15th day of fermentation (Batch 3, Fig. 6). It was also observed that on 13th day of fermentation, the peels had turned soft and orange peel skin

separated observed as layers (**Fig. 1c**) that enabled quick release of the oil from the peels. The fermentation beyond 15 days, however, led to browning of the peels, foul odour, and formation of black spores indicating dormant stage or exhaustion of medium and initiation of decline phase.

The 2 mm particle-sized orange peels produced mat growth of *Aspergillus sp./NCIM-1432* and caused softening of peels on 5-6th day of the fermentation and had a lag period of 4 days (**Batch 5, Fig. 6**). Thus the 2 mm particle size of the peels showed improved fermentation rate for orange oil production compared to 1x1 cm size peels.

The increased fermentation rate with reduced peel particle sizes can be related to the increased specific surface area available for the *Aspergillus species* to propagate on the orange peels. Torrado et al. [15], Bhargava et al. [46], Zadrazil and Puniya [47] have mentioned that in the case of *SSF* the mass transfer is influenced by the physical characteristics of substrate including moisture content, pore size and particle diameter.

The peels of particle size of 0.16 mm showed extended lag phase, produced foul odour and caused browning of the peels indicating the decomposition of peels. Thus, reduction in orange peel particle size below 2 mm did not give the desired results for orange oil extraction. The literature reports, including those Bhargava et al. [46], Torrado et al. [15] and Zadrazil and Puniya [47], have mentioned that unreasonable substrate size reduction leads to a decrease water holding capacity of the peels and hence decreases fermentation rate. Hence, it is crucial to optimize particle size of substrate for enabling efficient fermentation.

Effect of external nitrogen source

The peels contain only carbohydrates and lack proteins or nitrogen source which are also needed for the growth and development of the microbial cells [1, 11, 17- 18]. The same issue was faced in the present study owing to which an external nitrogen source was added. In a *SmF* with orange peel extract, conducted by Rangarajan et al. [48], peptone was used as the nitrogen source while soybean meal as the nitrogen source in *SSF* with orange peels using *Aspergillus niger*. An improvement in pectinase activity was reported due to the use of external nitrogen sources.

The impregnation of 2 mm average particle-sized orange peels with 2 % v/v tryptone solution showed an improved rate of fermentation. The lag phase was *only 2 days* and the seed culture showed the mat growth on the 5th day of fermentation (**Fig. 6**). Thus, the addition of an external In a *SmF* with orange peel extract, conducted by Rangarajan et al. [48], peptone was used as thenitrogen source while soybean meal as the nitrogen source in *SSF* with orange peels using *Aspergillus niger*. An improvement in pectinase activity was reported due to the use of external nitrogen sources. Thus, it can be stated that the addition of an external source of nitrogen increased the fermentation rate. In the present work too, the use of an external nitrogen source improved the fermentation rate (**Fig. 8**). Torrado et al [15] and Adebare et al. [49] had mentioned that nitrogen in the growth medium has a crucial role in microbial growth and enzyme production.

Effect of temperature

The fermentation of orange peel at 25 °C, 30 °C and 37 °C in petri plate produced brown coloured orange peels and the peels lost their freshness in 16 h. Further, the peels appeared dried and stale (**Fig. 9**). The colour of the oil and aqueous phase obtained by fermentation at these higher temperatures was brown. On the other hand, the fermentation performed at 8 °C, retained the freshness and colour of the peels. The oil recovered too was orange in colour.

Hence, the temperature of 8 °C was selected as the optimum temperature. Dhankher and Chauhan [50], have reported that change in the fermentation temperature has minimal or no impact on the product quality which contradicts the findings of the present research where a substantial change in product quality is seen with the change to higher temperatures. A similar effect of temperature has been reported in case of other products such as beer volatiles in the research work demonstrated by Kucharczyk and Tuszynski [51].

Depending on the fungal *species* used (*Aspergillus species* including *Penicillium atrovenerum*, *A. flavus*, *A. oryzae*, *A. niger*, *A.tubingensis*) for production of hydrolytic enzymes by either *SmF* or *SSF* the optimum fermentation temperatures varied from 30- 50° C. However, these works have not reported simultaneous extraction of the orange oil [15, 33, 47–49, 52-53]. All these studies tried the extraction process at relatively higher temperatures unlike the present research wherein to obtain a good quality of orange oil, the optimum fermentation temperature was 8°C.

However, this temperature (8 °C) was not optimum in case of enzyme activity. The enzyme activity at 8 °C temperature was determined to be 135 CMC.g⁻¹ and 88 IU.g⁻¹ for cellulase and pectinase, respectively. The respective enzyme activity at 25- 30° C are 223 CMCg⁻¹ and 126 IU g⁻¹ of cellulase and pectinase. The activities dropped at 50° C, to 121 CMC.g⁻¹ and 65 IU.g⁻¹ (**Fig. 9**). The enzyme recovered by the *SSF* of orange peels showed the maximum activity at 30 °C.

Extraction of fermentation products and their characterisation

Enzyme Activity:

The extraction of the oil from the aqueous phase was possible by manually compressing the fermented peels, followed by centrifugation at 200 rpm. However, the by-products, including cellulase and pectinase enzymes remained adhering to the peel surface. Hence, for the complete recovery of the fermented products, the fermented mass was washed with pH 5.5 citrate buffer solutions. Before moving on to removal of cellulase and pectinase enzymes from the fermented mass, it is important to detect the presence of these by-products.

Once the presence of cellulase and pectinase enzymes was detected, then the optimal pH value was determined so that the fermented mass could be washed with citrate buffer solutions of optimal pH to recover all the cellulase and pectinase enzymes. The pH has a direct impact on the activity of enzymes. It affects the ionization of the materials in the growth medium and influence enzyme production.

The extracted pectinase enzyme was quantified by iodimetric assay [22, 54- 55]. The solution showed the cellulase activity of 11.9 CMCg⁻¹ and pectinase activity of 8.5 IU.g⁻¹, respectively, if plain water was used. While the activity in 50 cm³ citrate buffer of pH 5 was 223 CMC.g⁻¹ of cellulase and 126 IU.g⁻¹ of pectinase. Thus, the extracted solution showed higher enzyme activities in the citrate buffer of pH 5.5. At other pH conditions, the enzyme activities were minimal.

The difference in enzyme activities in water and buffer is due to the pH. The pH maintaining ionic species, affects ionic conditions in solutions and change the active sites in the enzyme, and thus altering its activities [56- 57]. The studies conducted by Qasim et al. [58], Gangwar & Karthikeyan [55]; Dhembare et al [59]; show the optimum pH values (4 to 6) for hydrolytic enzymes extracted from *Aspergillus species* [55, 58- 59]. The optimum pH value for enzymes extracted from *Aspergillus*, is 5.5 which can thus be stated to be well within range of pH 4 to 6.

Rangarajan et al. [48] had reported a maximum of 4500 Ug⁻¹ of exo-pectinase activity and 500 Ug⁻¹ of endo-pectinase activity from an orange peel extract and dried orange peel as a substrate by *SSF* using *Aspergillus niger*, but reported no cellulolytic activity. Adebare et al. [49] reported the use of orange peels for production of cellulase with maximum endoglucanase activity of 30 U.cm⁻³ and pectinase with maximum polygalacturonase activity of 45 U.cm⁻³ using three different fungal species. Kannahi and Elangeshwari [33] reported 0.3 IU cm⁻³ and 0.62 IU.cm⁻³ of cellulase enzyme by *SmF* of orange peels using *Aspergillus niger* and *Trichoderma viridae*.

Mrudula and Anitharaj [1] reported maximum pectinase production of 1211.2 U.g⁻¹ by *SSF* of orange peels using *Aspergillus niger*. Sajith et al. [53] conducted a detailed literature review on cellulase enzyme production by *SmF* using different fungal strains and have found that the enzyme activities which ranged from 1.6 Ucm⁻³ till 2793 Ucm⁻³ and in case of *SSF* the cellulase activities ranged from 2 U.gds⁻¹ to 5408.5 U.gds⁻¹.

It can be thus stated that enzymes work best within specific temperature and pH ranges, and sub-optimal conditions can cause the enzyme to lose its ability to bind to a substrate. It can be inferred from the literature above that the enzyme activity ranges over a broad spectrum from as low as 0.3 IU cm⁻³ to as high as 2793 Ucm⁻³ (for cellulase) and from 45 U.cm⁻³ to 1211.2 U.g⁻¹ (for pectinase). The enzyme activity found in the current research (cellulase activity of 11.9 CMCg⁻¹ and pectinase activity of 8.5 IUg⁻¹, in water while in citrate buffer, 223 CMCg⁻¹ of cellulase and 126 IUg⁻¹ of pectinase) (**Fig. 10 b**) falling in the ranges reported by other researchers.

Enzyme Kinetics

The enzyme kinetics plays an important role in analyzing the enzyme efficiency. The V_{max} value of the cellulase enzyme was 7.31 x 10⁻⁵ (mg cm⁻³) s⁻¹, and its K_m value was 6.3 mg. The V_{max} value of the extracted pectinase enzyme was 3.2 x 10⁻⁴ (mg cm⁻³) s⁻¹, and its K_m value was 3.8 mg (**Figure 9 c-d**). The reported K_m value for pectinase, in the existing literature are in the range of 0.0201 – 4.22 mg.cm⁻³ and the V_{max} values for the pectinase are reported as 6.6 x 10⁻⁴ mole.cm⁻³s⁻¹ [60]. The reported V_{max} values for the cellulase are 2.89 x 10⁻⁴ mg.cm⁻³.s⁻¹ to 6.94 x 10⁻⁴ mg.cm⁻³.s⁻¹ and the values for K_m is 0.28 % CMC [61]. The K_m determine the affinity to the substrate and the V_{max} values indicate the kinetic efficiency of the enzyme. The enzymes generated in the *SSF* process indicated moderate affinity and moderate rate of reaction. The reason for such behaviour can be because of the inhibitory effects of other fermentation products such as the sugars.

Enzyme Purification and Recovery

The enzyme purification at 0- 4 °C prevented enzyme denaturation. The ammonium sulphate precipitation produced enzyme activities as given in **Table 1**. The specific activity increased with increasing ammonium sulphate concentration. The saturation of the solution with the salt improved the protein-protein interactions and caused their agglomeration while improving the specific activity of the protein in the precipitate.

The dialysis step removes the excess salt from the enzyme solutions. The fractional precipitation of the enzyme solution with ammonium sulphate (0-80 % w/w) gave the enzyme activities of 242 CMC g⁻¹ and 178 Ug⁻¹ for cellulase and pectinase, respectively. The purification factor was 5.25 and 10.86 with 53.3 % and 49.2 % recovery of pectinase and cellulase. The remaining enzymes were not recoverable, and the process requires modification

Spectro-flurometry

The emission spectrum of ammonium sulphate precipitated enzyme solution showed typical peaks after excitation with UV light at 280 nm. Tryptophan shows the emission wavelength between 300 - 350 nm with prominent fluorescence peak at 342 nm (**Fig. 10**). The active sites of the cellulase consist of amino acids including tryptophan. The indole ring of tryptophan possesses an internal fluorescence activity [25].

SDS-PAGE Chromatography

The reducing The crude enzyme sample and ammonium sulfate precipitated enzyme samples when resolved on SDS- PAGE reducing gel, showed two prominent bands at 30 and 55 kDa (**Fig. 9**).

The molecular weights for the extracted enzymes are given in **Fig. 9**. The reducing SDS-PAGE forms the subunits of a protein and to know the actual molecular weight native PAGE or size exclusion chromatography is further required. Since the chief motive of the study was orange peel oil extraction, the actual molecular weight native PAGE has not been considered. The literature reports indicate that the molecular weights of the enzymes vary with the source of the enzyme. *Aspergillus niger* has shown pectinase enzyme with a molecular weight of 30 kDa on reducing SDS-PAGE [62] and endoglucanase (cellulase) from *T. viridaea* has shown molecular weight close to 39.2 kDa [7]. In the present research, the molecular weight, as determined by SDS-PAGE, is around 30 and 55 kDa, that is almost identical to molecular weight well characterized by Pirzadah et al., [62] and Ahmed et al., [7].

3.10 Characterization of extracted orange peel oil

The GC-MS analysis of the oil showed the presence of α -pinene, d-limonene, limonene oxide, linalool and citronellal in the extracted oil. The composition of oil is comparable to the composition of oil obtained by supercritical extraction process [14] by our processes of UAE and MWAE assisted extraction) The HPLC-MS showed the presence of Tangeritin, Sinensetin, Nobiletin, Tocotrienol, and Tocopherol acetate in the oil. The composition of the oil is comparable with different processes in literature [63-65].

Further, figure 11 is a graphical representation of growth of the fungi studied based on the weight difference of the initial fermentation medium and that obtained with fermentation growth on respective days.

The fermentation of orange peel has been reported for production of cell wall hydrolysing enzymes including cellulase, pectinase [15, 33, 47–49, 52-53]. The orange oil extraction using solvent extraction method, hydro-distillation method [66], ultrasound assisted extraction [67], and cold press combined with enzyme extraction method [68], has been also reported in the literature. However, the fermentation process reported in literature for enzyme production does not report oil extraction. Also, unlike the reported orange peel oil extraction methods, the current method is devoid of using any heat treatment or sophisticated high pressure equipment or drying or peels.

Conclusions

The extraction of orange peel oil was conducted by treatment with *in-situ* generated extracellular enzymes secreted by fungi during *SSF*. Owing to the utilisation of the by-product enzymes for the process of extraction of oil, the method can be termed as a green method. The fermentation parameters included in the process of extraction are sterilization technique (70 % v/v alcohol), fermentation temperature (8 °C), external nitrogen source (2% w/v tryptone), particle size (2 mm), and inoculum size (10^4 fungi cm^{-3}). During the process of development of the green method, a strain of *Aspergillus species*/ NCIM -1432 has been isolated. *Aspergillus species*/ NCIM -1432 has been aiding in thorough extraction (~ 0.95 % w/w) of good quality orange oil. The ammonium sulphate precipitated solution had 178 IU.g⁻¹ and 435 CMC.g⁻¹ pectinase and cellulase activities, respectively, at optimum extracting buffer pH of 5.5, and in the 25-30 °C extraction temperature range. A green, sustainable, alternative to the present extraction methods has thus been developed for the extraction of orange oil. The method enables the production of enzymes as a by product during the process. However, the fermentation need longer time for producing the results compared to extractions processes which is its major limitation.

Abbreviations

BLAST	Basic Local Alignment Search Tool
CMC	Carboxy methyl cellulose
FDA	Food and Drug Administration
FAE	Fermentation assisted extraction
NA	Nutrient agar
NB	Nutrient broth
NCL	National Chemical Laboratory
SSF	Solid-state fermentation
SmF	Submerged fermentation
US	United States
FAE	Fermentation assisted Extraction

Declarations

Ethics approval and consent to participate: The work does not involve any animal or human participants

Consent for publication: YPL and VGG are willing to publish the work

Availability of data and materials: Data was generated in Department of Chemical Engineering, Institute of Chemical Technology and materials were obtained from the chemical suppliers in India

Competing interests: No competing interest

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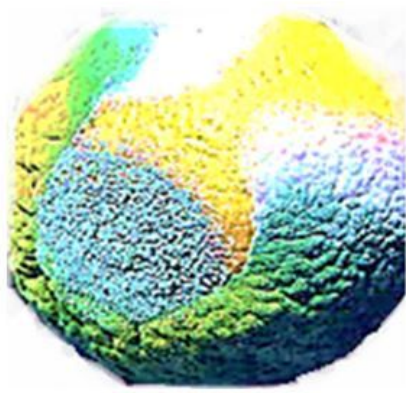
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Figures



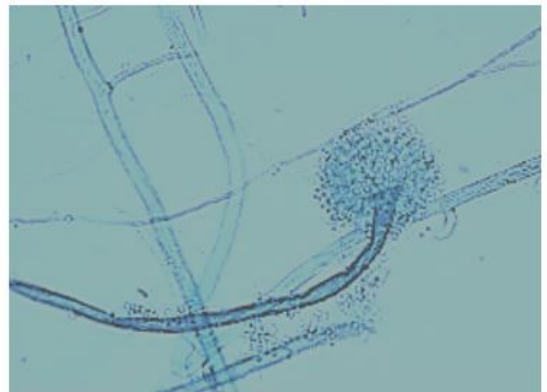
(a)



(b)



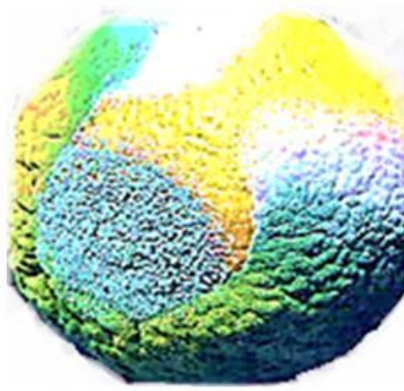
(c)



(d)

Figure 1

a Macroscopic appearance of the different coloured fungal colonies on orange peel, b Macroscopic appearance of selected fungal culture on the orange peels, c Orange peel skin separated as layers d Microscopic image of isolated *Aspergillus* sp. (45 X)



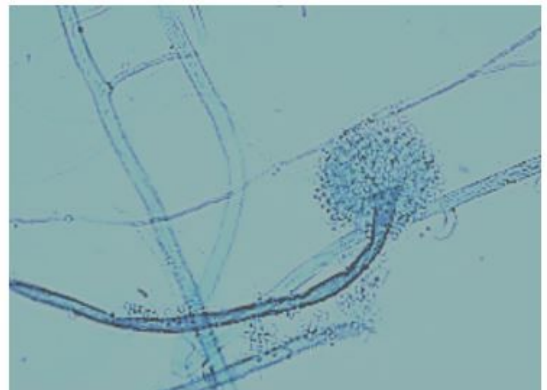
(a)



(b)



(c)



(d)

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(a)



(b)



(c)



(d)

Figure 2

The oil obtained by squeezing the peels with (a) mixed culture, (b) white filamentous growth, (c) green growth, (d) black growth



(a)



(b)



(c)



(d)

Figure 2

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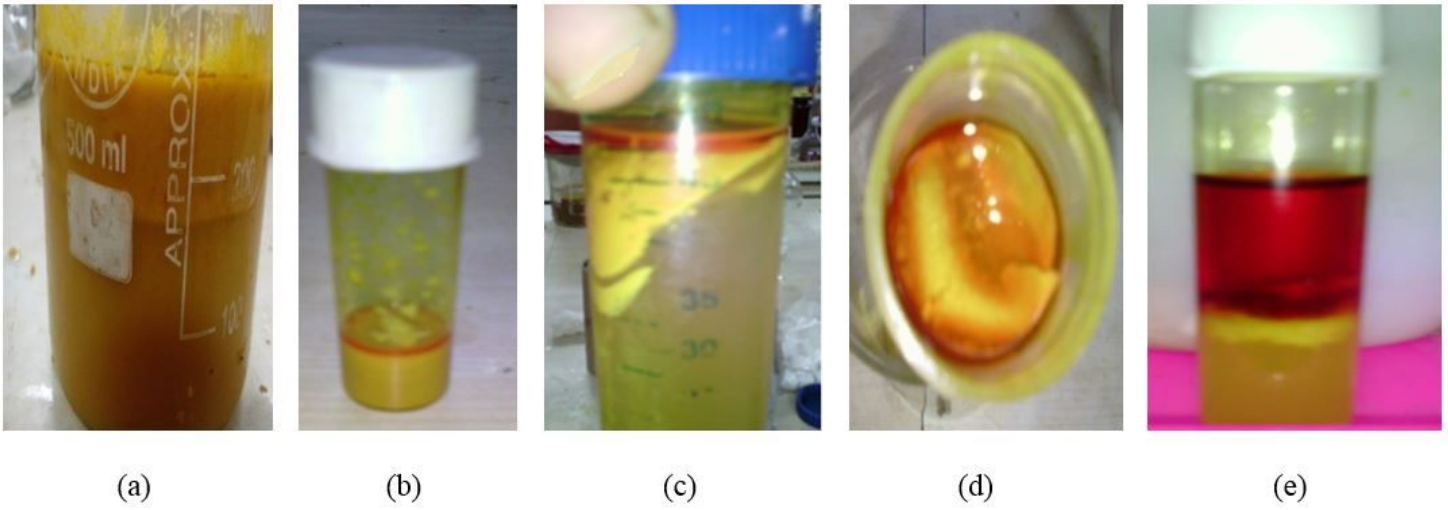


Figure 3

Appearance of the extracted oil from the isolated *Aspergillus* species a The aqueous phase after squeezing, b Oil collected by squeezing, c-e Oil collected after centrifugation

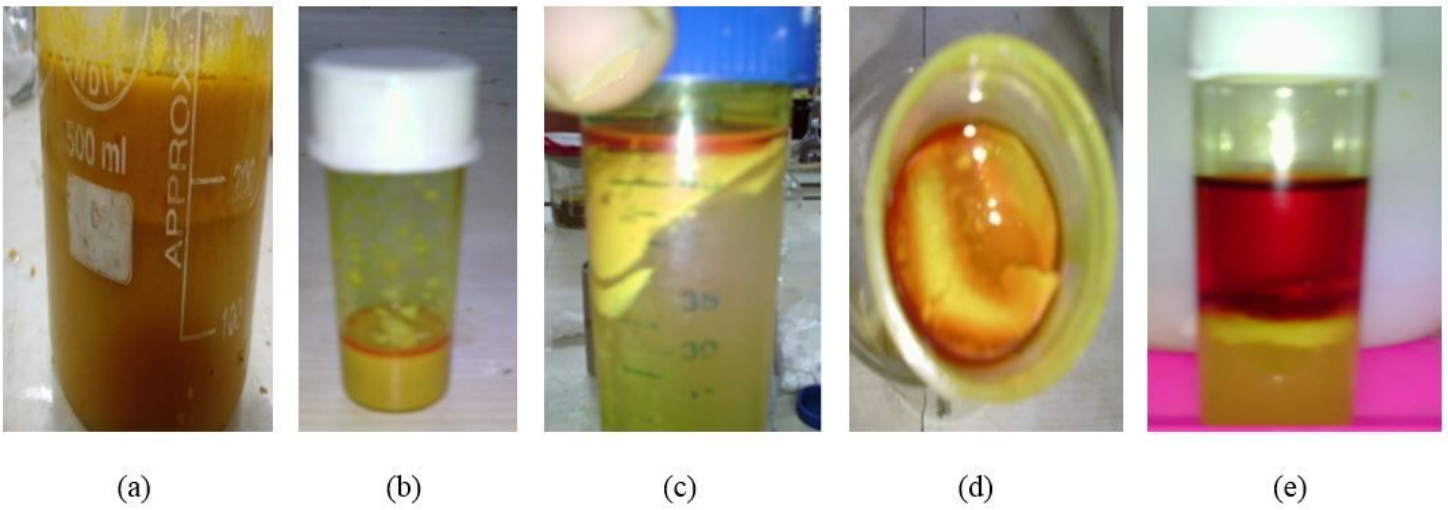
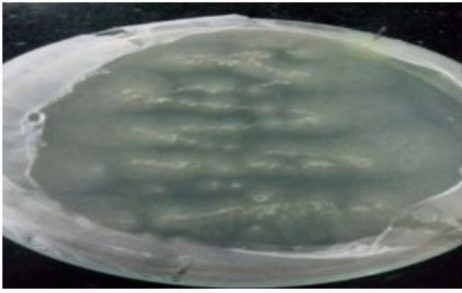


Figure 3

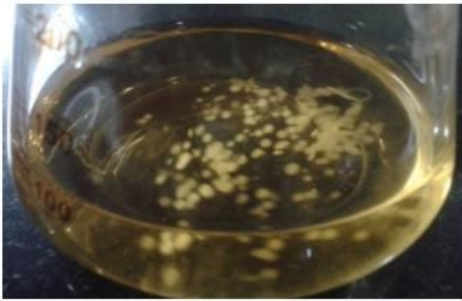
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(a)



(b)



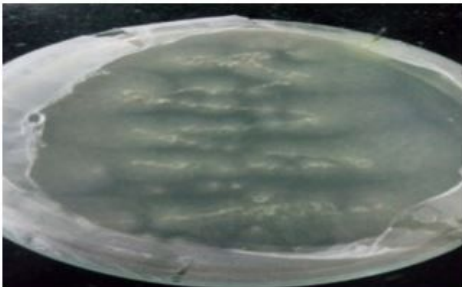
(c)



(d)

Figure 4

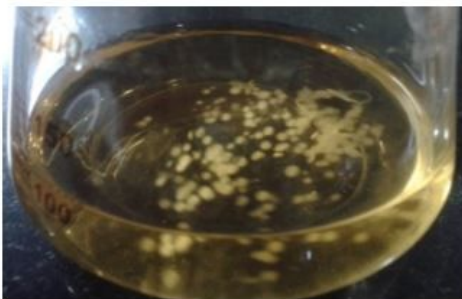
a Seed culture development studies on the nutrient agar plate at fermentation period of A.12 h, b 24- 72 hand Seed culture development studies in nutrient broth at fermentation period of c 72 h, d 96- 108 h



(a)



(b)



(c)



(d)

Figure 4

a Seed culture development studies on the nutrient agar plate at fermentation period of A.12 h, b 24- 72 hand Seed culture development studies in nutrient broth at fermentation period of c 72 h, d 96- 108 h

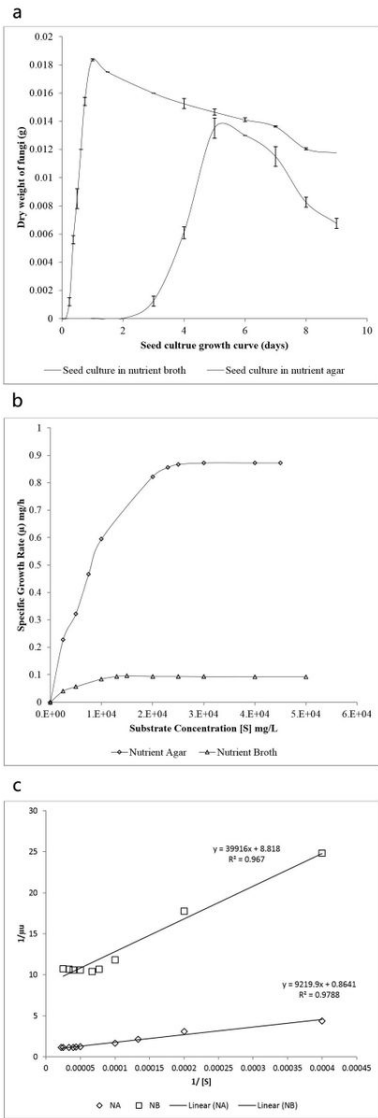


Figure 5

a: Growth curve of seed culture in Nutrient Broth: (Peptone: 0.5%, Beef extract/Yeast Extract: 0.3%, NaCl: 0.5%, Agar: 1.5% - 15g/L-1, 1 % carboxy methyl cellulose) and in Nutrient Agar: (Peptone: 0.5%, Beef extract/Yeast Extract: 0.3%, NaCl: 0.5%, Agar: 1.5%, 1 % carboxy methyl cellulose -30 g.L-1) b: Monod kinetics: Growth rate of the selected *Aspergillus* species seed culture in Nutrient Agar and Nutrient broth medium containing 1 % carboxy methyl cellulose c: Inverse of specific growth rate v/s inverse of substrate concentration

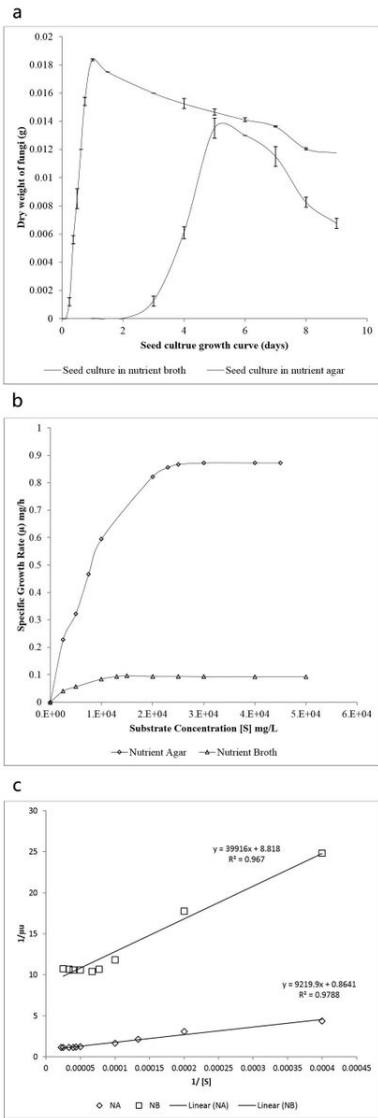


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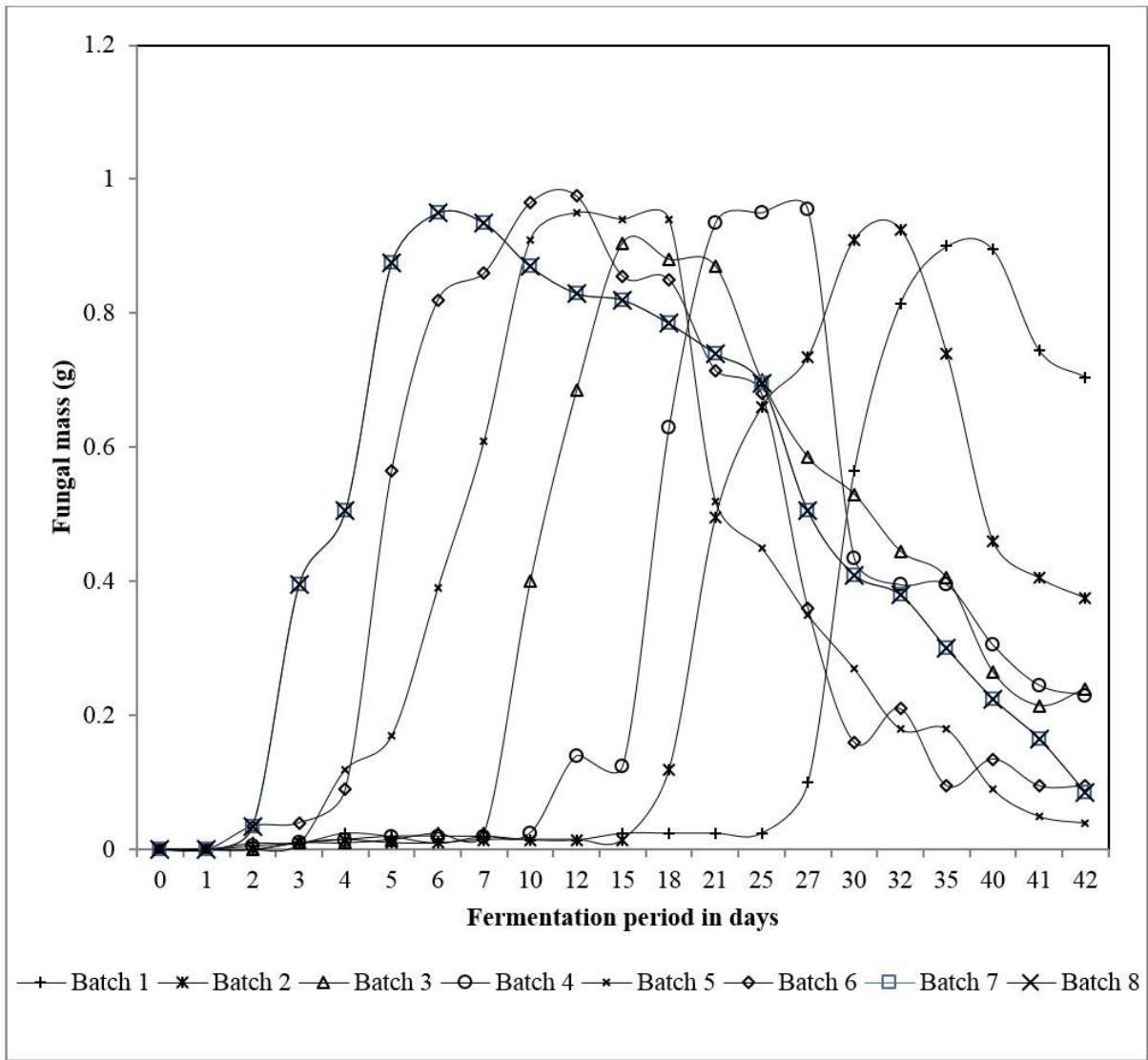


Figure 6

Growth Curve of fungi: Growth of the fungi based on the weight difference of the primary fermentation medium and that obtained with fermentation growth on several days. (Batch 1: 1x1 cm orange peel, 101 fungi. cm-3, Batch 2: 1 x1 cm orange peel, 102 fungi cm-3, Batch 3: 1x1 cm orange peel, 104 fungi.cm-3, Batch 4: 1 x 1 cm orange peel, 106 fungicm-3, Batch 5: 2 mm orange peel, 104 fungi cm-3, Batch 6: 2 mm orange peel, 104fungi cm-3, 1 % w/v tryptone, Batch 6: 2 mm orange peel, 104 fungi cm-3, 2 % w/v tryptone, Batch 6: 2 mm orange peel, 104 fungi cm-3, 3 % w/v tryptone).

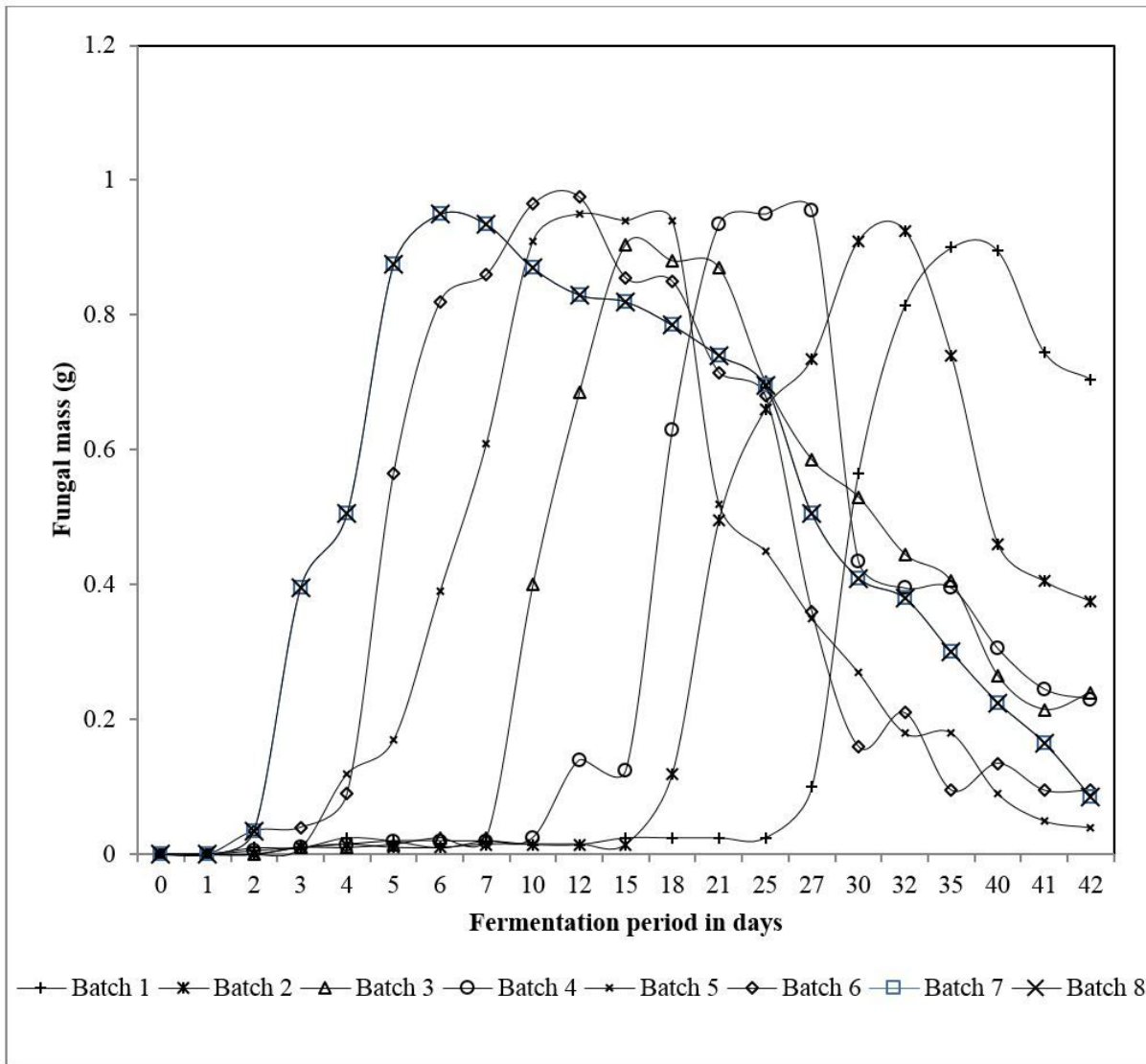


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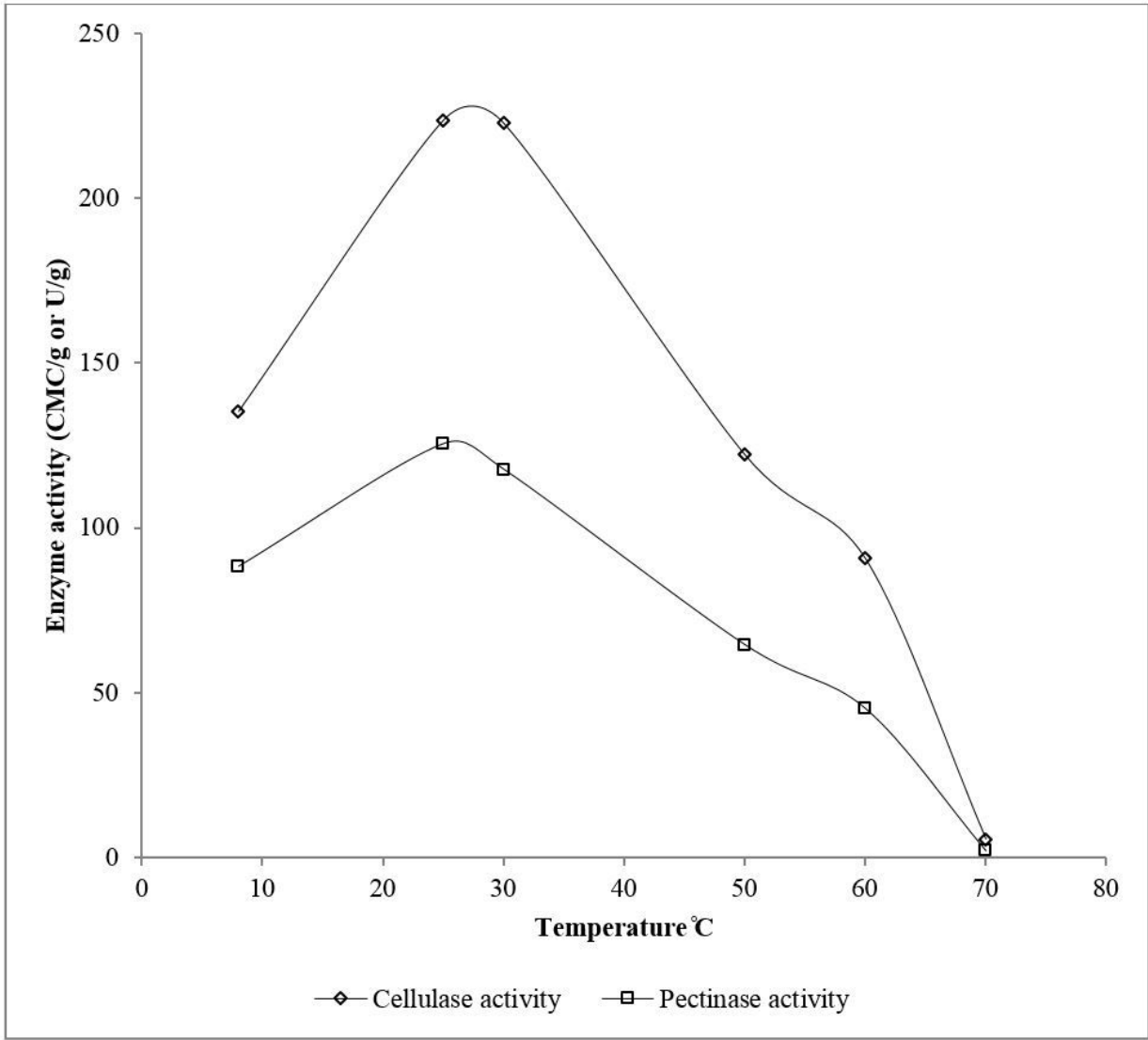


Figure 7

Effect of Temperature (°C) on Enzyme activity.

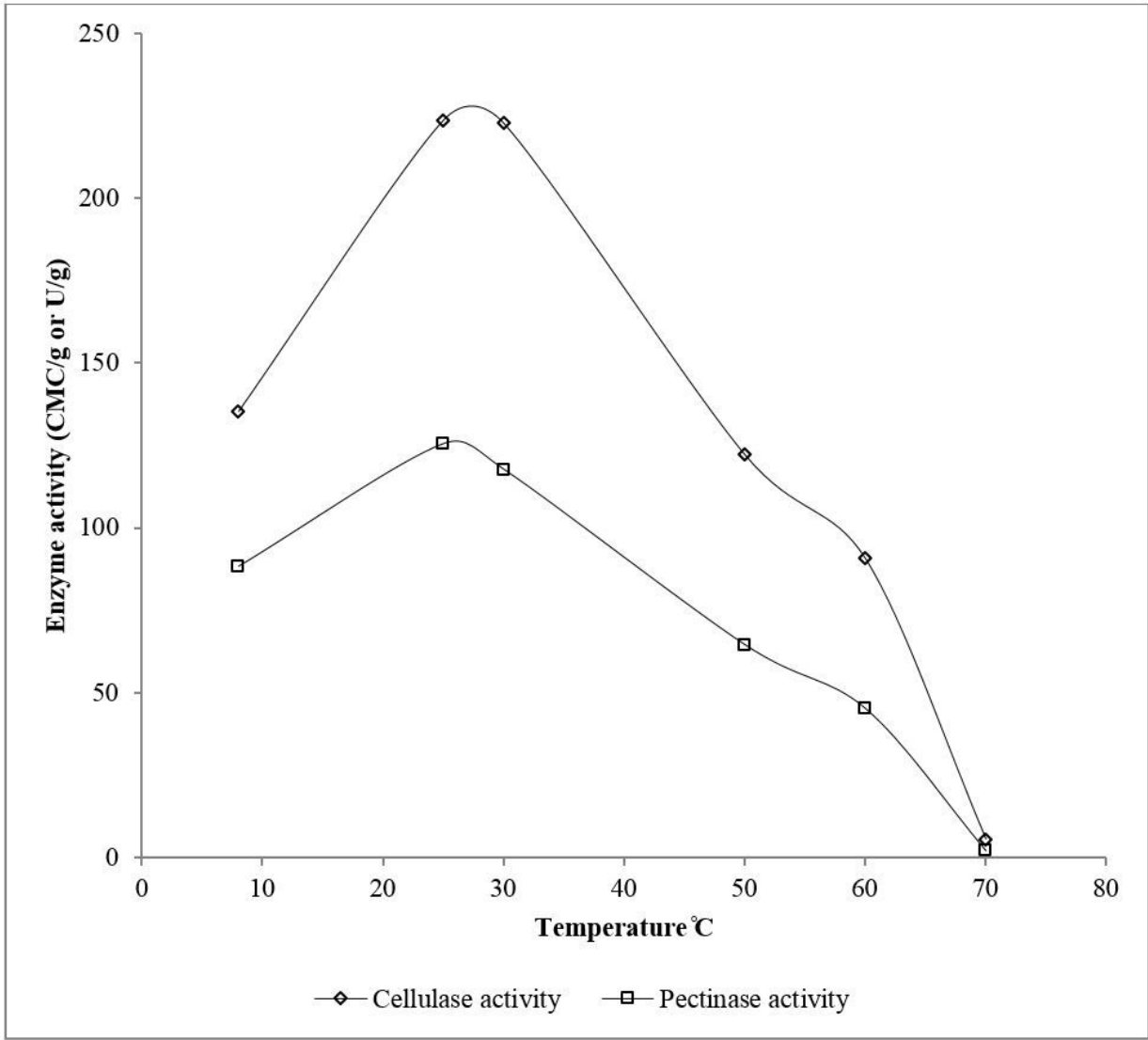


Figure 7

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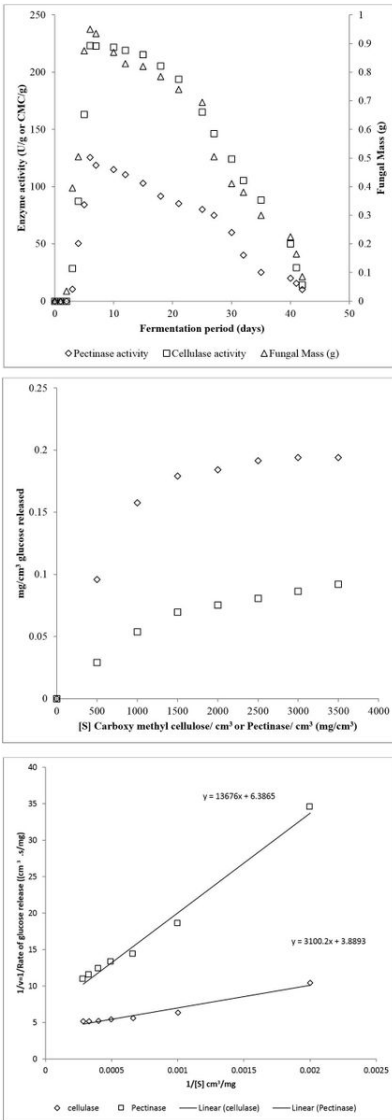


Figure 8

b Enzyme activity and weight of fungal mass with fermentation period in days determined at optimum enzyme temperature (25-30°C), pH 5.5, 104 fungi.cm-3, 2 % v/v tryptone concentration. c Characterization of extracted enzyme. Plot of glucose released v/s substrate concentration, ◻- Cullulose, ◊- Pectin, S: Substrate Concentration. d Characterization of extracted enzyme Plot of inverse of glucose release per unit time v/s inverse of substrate concentration.

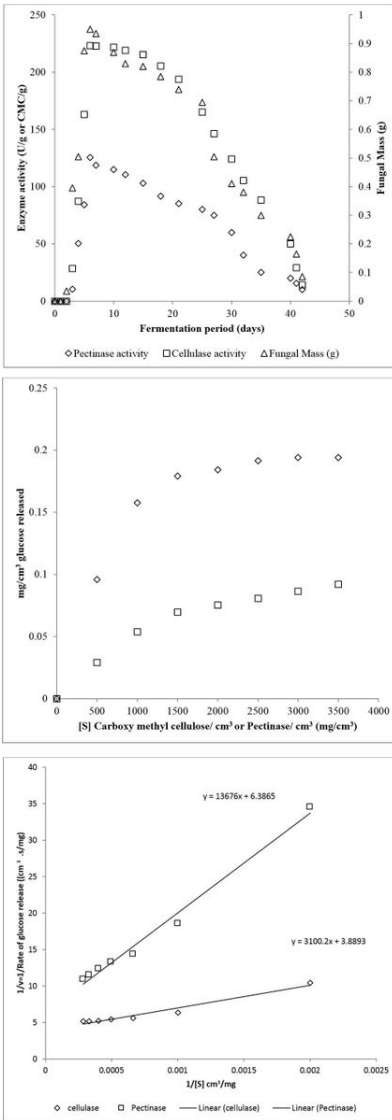


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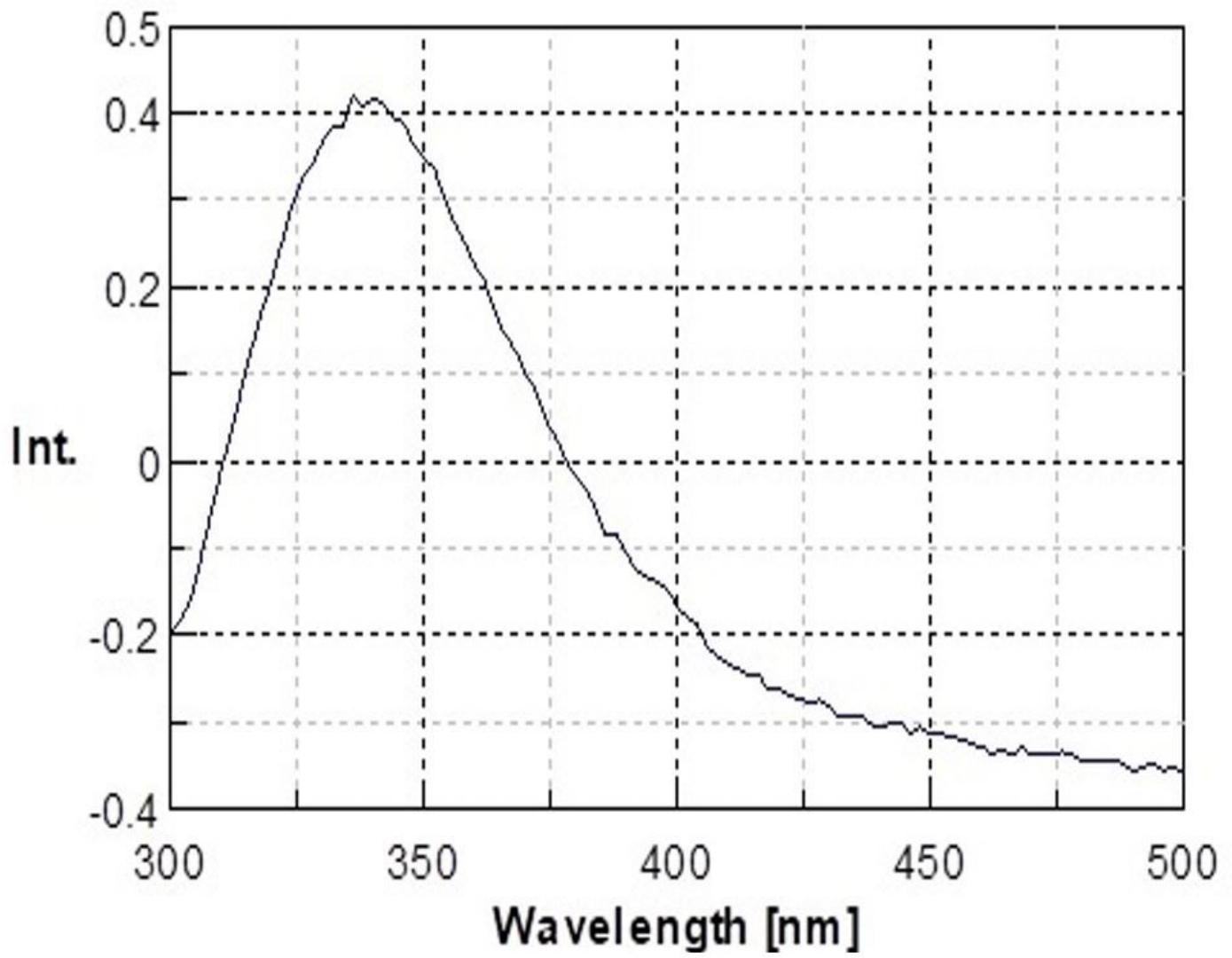


Figure 9

Emission Spectrum of enzyme extract after ammonium sulphate precipitation

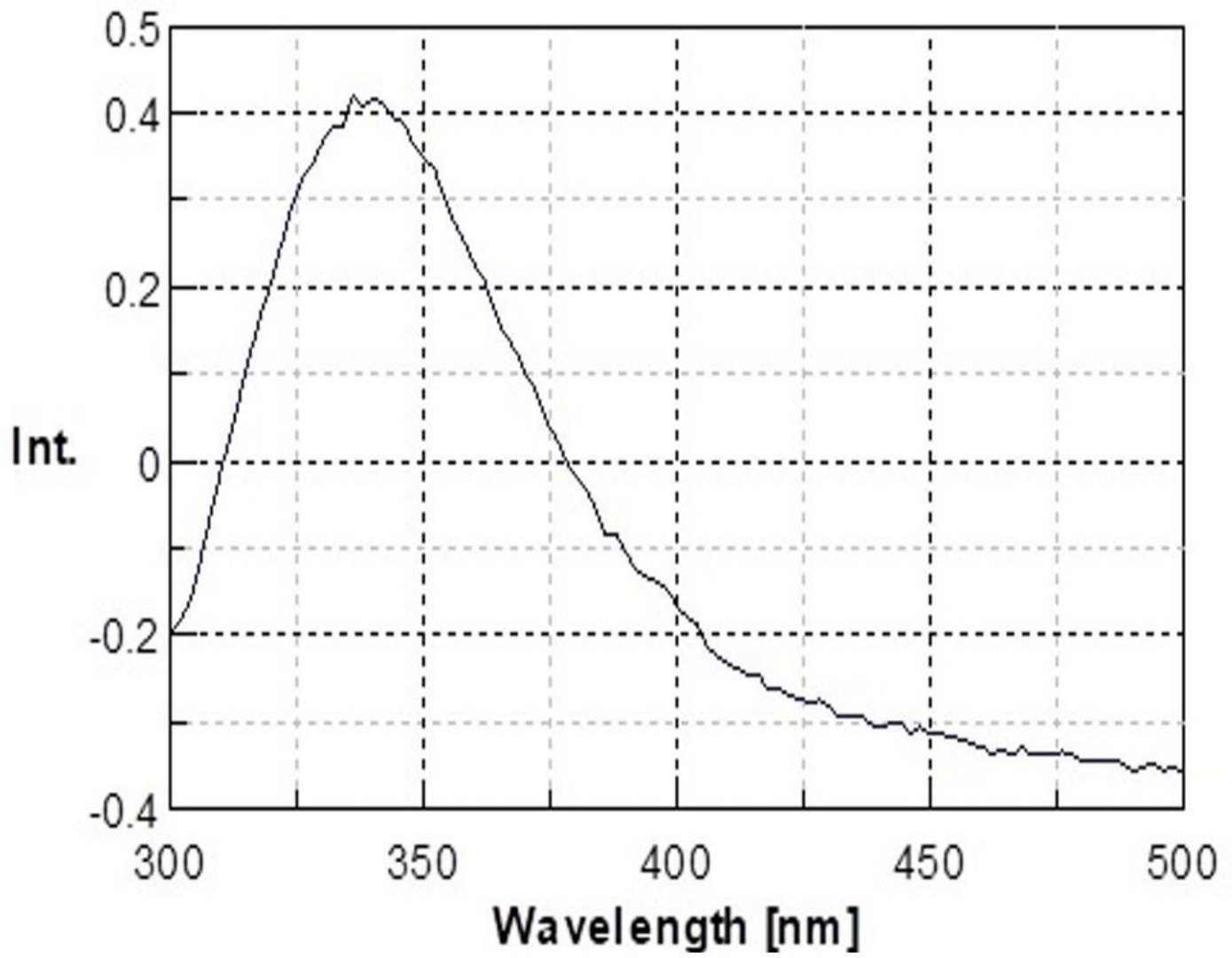


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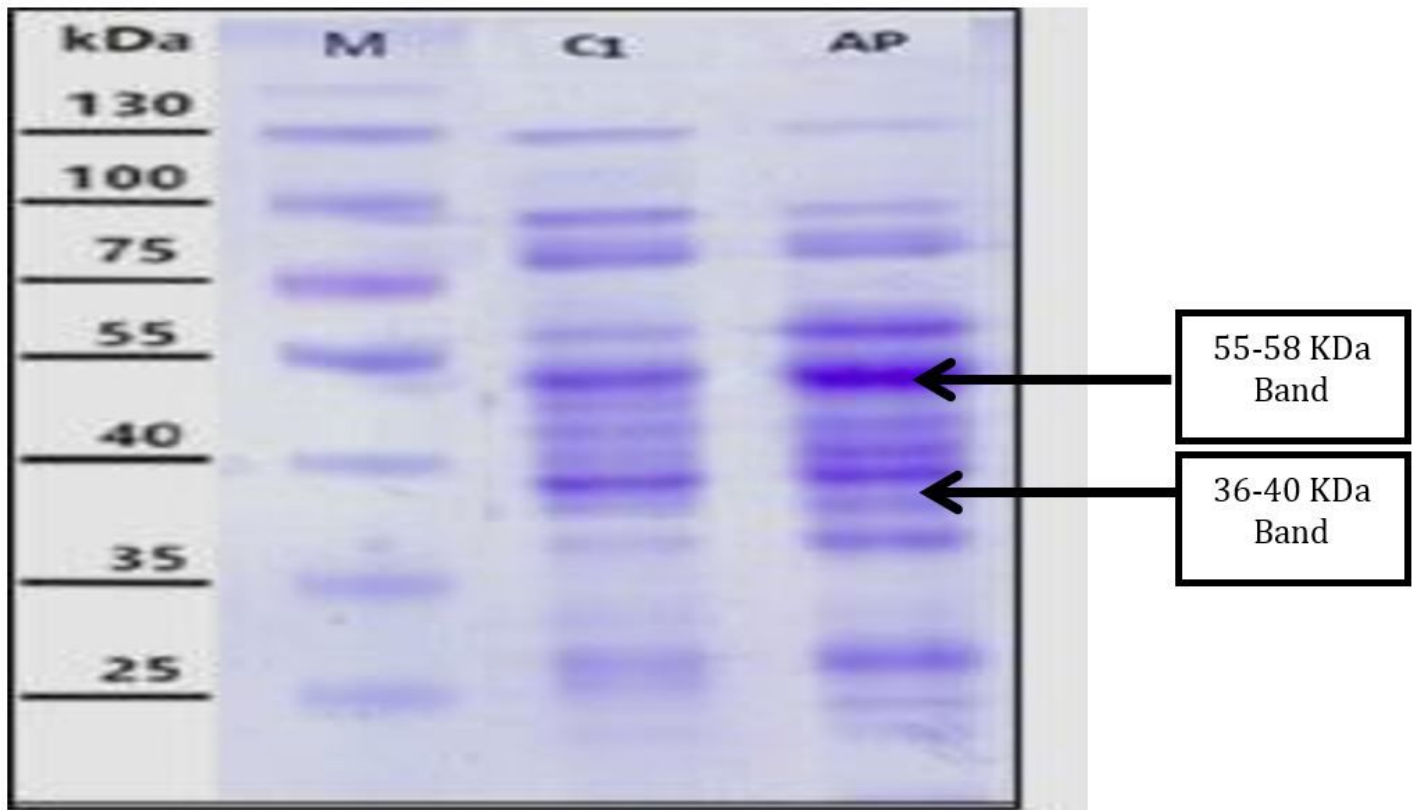


Figure 10

SDS-PAGE of the aqueous phase of fermentation assisted oil extraction M: Molecular weight marker, C1: Crude of FAE, AP: Ammonium sulfate precipitation of the crude

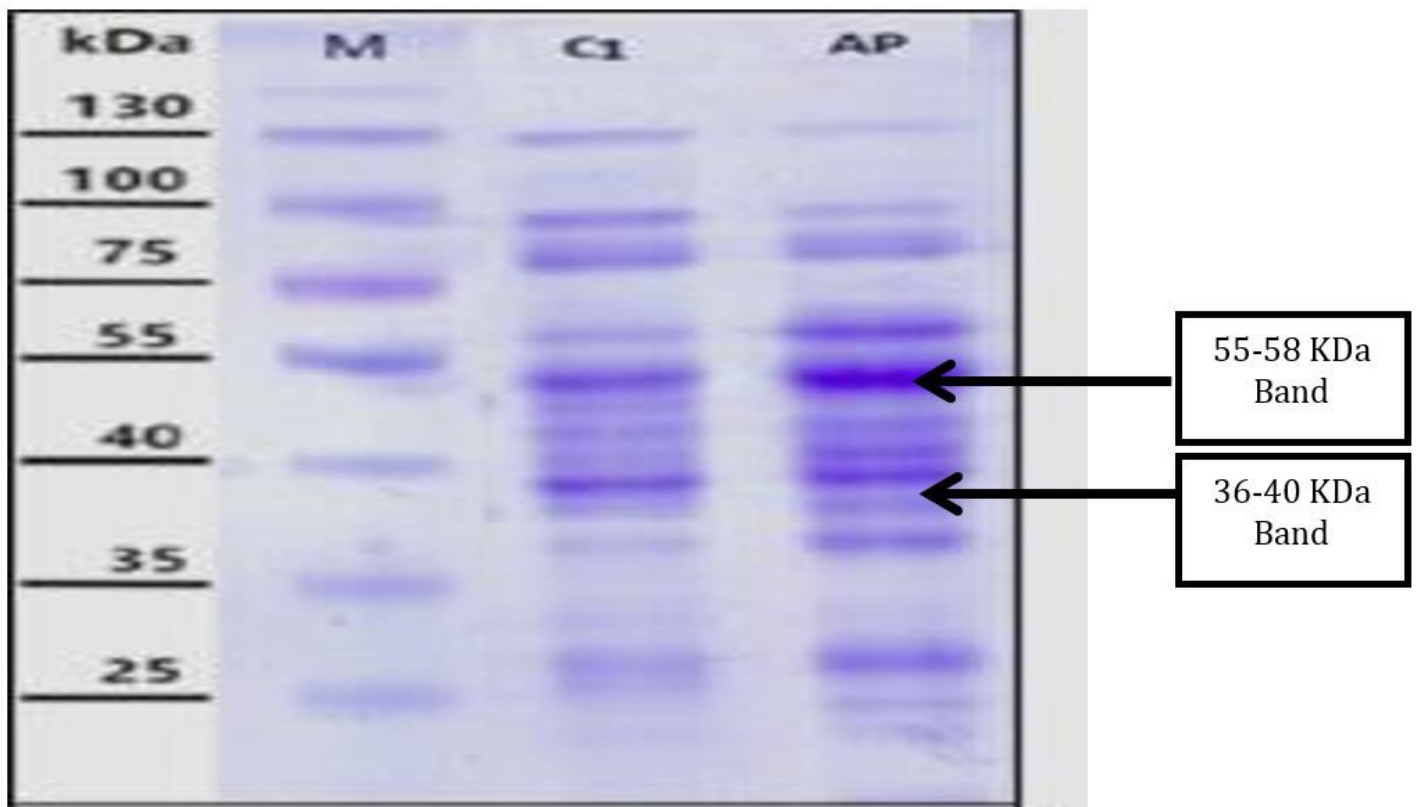


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