**Solid-State Fermentation of Orange Peels for Recovery of Orange Oil** **using *Aspergillus species* NCIM 1432**

**Abstract**

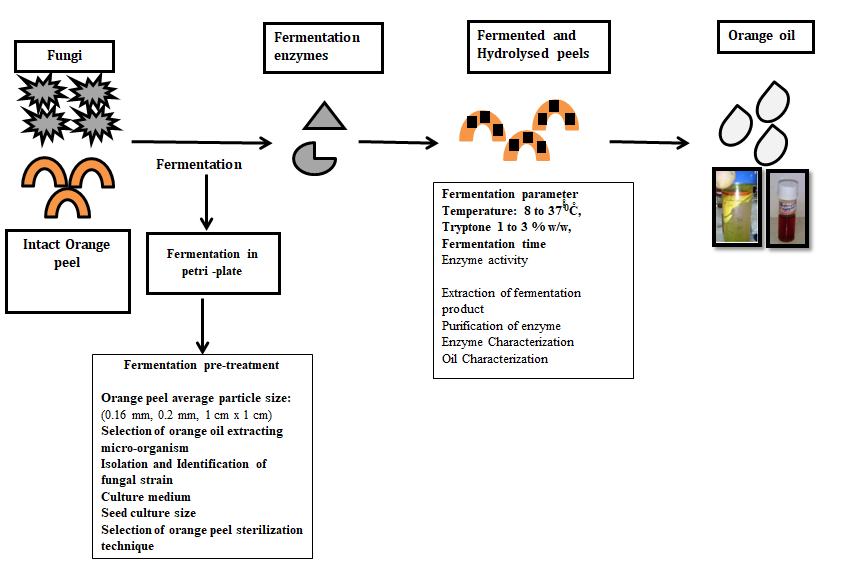
The available orange peel extraction processes have several drawbacks including the need of solvents and high temperatures which affect the quality of orange oil. Thus there is a need to develop an alternate green process for extraction of orange oil. In present study, solid state fermentation (SSF) of fresh orange peels by *Aspergillus species* NCIM -1432 to extract extra-cellular enzymes and orange peel oil are noted. Additionaly, the effect of methods of orange peel sterilisation, importance of selection of the fermentation strain, effect of external nitrogen source, and particle size of the peels are detailed.

The process used here gave 0.95 % w/w yield of orange oil. The color of the extracted oil is noted with 56.8, 4.7 and 12.2 CIE L\*a\*and b\* values respectively typical to the orange colour. Also, the oil has been noted with typical orange fruit aroma. Further, the chemical composition of the extracted oil has been monitored by GC-MS, HPLC –MS which showed 96 % w/w limonene content. The oil is noted with anti-oxidant property upon its DPPH assay, with 0.87 of specific gravity. All the properties of the oil indicate a good quality oil. The crude hydrolytic enzymes produced during fermentation showed 155 U. g-1 and 239 CMC. g-1 of pectinase and cellulase activity respectively.

The developed method of orange oil extraction is green, needs low tech equipment and can be an alternative to the available orange peel oil extraction methods at small scale.

**Keywords:** Functional foods, Food processing techniques, Essential oils in food, Bioactive compounds, Flavor compounds, Citrus by-product utilization

**Graphical Abstract**

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1. **Introduction**

India ranks 64th with regard to the production of oranges world-wide. There is 9.23 tonnes per hectare prouction of oranges which corresponds to around 29 lakh tonnes of oranges. Thus, 40 % of the total area in India is under citrus cultivation [1].

Only the pulp portion of orange fruit i.e. 70% w/w portion of the fruit is consumable due to the great taste. The 30 % portion of the orange fruit which comprises of orange peels and seeds are generally regarded as wastes and are discarded as such. Thus, annually a large amount of orange by-product is generated as waste including orange essential oil, seeds and carbohydrate powder. The orange fruit waste has many applications in pharmaceutical, food, cosmetic industries due to its anti-microbial, anti-fungal, insecticidal properties [2]. Discarding the orange waste as such can be a loss of valuable resource for agro-industry also, discarding the orange waste as such has a serious impact on the environment [3].

There are several reports on the extraction of orange essential oil by organic solvent [4], thermal evaporation or distillation or hydro distillation [5], steam distillation [6], steam explosion [7], mechanical expulsion [8], supercritical carbon-di-oxide (*sc*CO2) [9], ionic liquid [10], hydrotropes [11] and by enzyme solution [12], [13]. The quality of the oil produced and that of the peel residue obtained is related to the process used for oil extraction. The conventional oil extraction methods are energy intensive [14]. The oil extracted by the mechanical press, enzyme treatment, and with *sc*CO2 is usually of high quality [9, 13, 15]. But the oil yields obtained by the mechanical press are low in amount, the extraction by supercritical fluids need expensive high-pressure equipment and has a scale-up issue. At the same time, the use of organic solvent produces good yields of oil, but the quality of the oil is affected due to residual contamination by the solvent and the temperatures required for the process, and also the solvent recovery is tedious [4, 9- 10, 12, 15, 16].

Owing to the commercial importance of the essential oil and the remaining by-products of orange peel, also due to the several drawbacks observed in the available orange peel oil extraction methods there is a need to develop an alternative green, sustainable method for orange peel oil extraction [8, 17-18].

Further as the enzymes have been utilized 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 the recovery of oil from the orange peels is facinating. The methods including submerged fermentation (*SmF*) or solid-state fermentation (*SSF*), are more commonly followed for recovering enzymes using agricultural waste. Mrudula and Anitharaj [19] reported the production of pectinase enzyme by *Aspergillus niger* using orange peel as the substrate. Mamma et al. [20] has reported the production of pectinolytic, cellulolytic and xylanolytic enzymes using mesophilic *Aspergillus* niger*, Fusarium* oxysporum*, Nwuraspora* crassa*,* and *Penicillium* decumbens under *SSF* conditions. [21]

Schuster et al.[22] have reviewed *Aspergillus* niger to produce extracellular enzymes for bio-transformation of orange peel 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. Rivas et al. [23] and Ahmed et al. [24] 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 the *SmF*, the dry orange peel powder or orange peel hydro-lysate or liquor are used in 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 [25] [26]. Achappa et al., [21] have mentioned about extraction of cellulose enzyme using solid state fermentation.

Additionally, it is challenging to store the orange peels as the peels are susceptible to microbial degradation due to their high moisture and carbohydrate content. The moisture and carbohydrate content in the peels, supports the microbial growth, while 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, and as the limonene *free* orange peel are suitable for the cattle feed [27]. Omran et al. [28] have separated the orange oil by distillation before fermentation of peels with *Aspergillus* species.

Suneetha et al.[29] and Wikandari et al. [30] have mentioned the advantages of *SSF* over *SmF*. *SSF* has been described as the process in a solid matrix (inert support or support/substrate) which provides an anchorage for the microbial cultures and also prevents bacterial contamination due to low moisture content. *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 the *SSF* mechanism make it a method of choice in the present research.

There are several reports on the 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 [9], [31], [32], [33], [34].

The literature indicates that *SSF* assists in enzymes, antibiotics, organic acids, pesticides, aroma, and bio-fuel production. However, there are no reports on orange peel oil extraction by generating enzymes by fermentation [30], [35], [36]. Based on ability of the fungal hyphae to penetrate the polymeric structure of peel, absorb the nutrients from the peels and produce the enzymes that hydrolyze the cell wall which can enable the extraction of orange oil. The current work uses *SSF* of orange peels to enable extraction of orange oil.

1. **Materials and Methods**

The study does not involve any human or animal figures. 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 solvents such as n-hexane, acetonitrile, methanol of AR Grade were obtained from SD. Fine Chemicals Ltd., Mumbai. The standard protein markers of 10 to 100 kDa. range was procured from Fisher Scientific and Microfilters (0.2 µm), an ultrafiltration membrane of 100 KDa from Millipore Pvt. Ltd., while dialysis membrane (24 ̊A) was purchased from Thermo Fisher Scientific.

**2.1 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 (0.16mm to 2 mm). The chopped peels of different particle sizes (5mm) were weighed to prepare a 100 g orange peel per fermentation batch. Each batch was repeated minimum three times.

**2.2 Selection of orange oil extracting Microorganism**

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 of different colours (brown, green, black). The colony that enabled the production of orange peel oil with characteristic organoleptic properties was selected for further oil extraction by the fermentation process [37].

**2.3 Inoculum Preparation:**

The microorganism was cultured on potato dextrose agar (PDA) slants at 28°C for 7 days. Spores were harvested by adding 10 mL of sterile distilled water with 0.1% Tween 80 to each slant. The spore suspension was filtered through sterile cheesecloth and adjusted to 1 × 10^7 spores/mL using a hemocytometer. This standardized suspension was used to inoculate the sterilized substrate at 2% (v/w) for solid-state fermentation.

**2.4 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.

**2.5 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 101 fungi cm-3 to 106 fungi cm-3 was studied to find an optimum seed culture size. The growth curve was determined with minimum of three replicates 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.

**2.6 Screening of orange peel sterilization technique**

To enable propagation of single fungal colony on fresh orange peels, three sterilization techniques were adopted. Firstly, the **High-pressure steam sterilization method** in an autoclave, operated at 121 0C, 15 psi pressure for 15 min. The second is **UV light irradiation treatment** in a 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 a sterile container.

**2.7 Optimization of Fermentation parameters**

Only fresh and sterile orange peels were used for fermentation in a 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) was investigated on the extraction of oil, production of enzymes and fermentation period with minimum of three replicates.

**2.8 Monod Kinetics:**

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

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

(1)

(2)

Where, Cf: Final microbial concentration, Co: Initial microbial concentration, k: specific growth rate/ rate constant, tf: final fermentation time, to: initial fermentation time

**2.9 Extraction of fermentation products**

The oil and enzymes produced in each fermentation batch were extracted from the fermented orange peels by manually pressing the peels in 50 cm³ of either deionized water or 50 mM citrate buffer solutions with pH values ranging from 5.0 to 5.5. The oil separated and formed a surface layer, which was collected by aspiration from the top. The remaining solid and aqueous phases were first filtered using a strainer and then centrifuged at 200 rpm for 5 minutes. The peel residue was subsequently washed three times with 10 cm³ of citrate buffer at the optimized pH of 5.0 to recover the enzymes from the fermented peels.

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

**2.10 Purification of enzymes**

A volume of 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. The final mixture was left for 16 h stirred continuously on a magnetic stirrer to allow complete precipitation of proteins. The suspension was centrifuged. The solid pellet was dissolved in 10 cm3 of 50 mM citrate buffer (pH 5.5) and dialyzed 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 analyzed for enzyme activity.

**2.11 Purification** **factor** (PF) or the fold purity after ammonium sulphate precipitation was defined as the relation between the specific activities of the enzyme (U/mg) in the resuspended precipitate to the specific activity of crude enzyme (U/mg), respectively.

Percentage recovery of the enzyme was measure by using the amount of enzyme activity obtained post ammonium sulphate precipitation to that obtained as crude enzyme.

**2.11 Enzyme Characterization**

All enzyme activities were assayed in triplicate using 50 mM citrate buffer of pH 5 unless noted otherwise. The zone of inhibition study on CMC agar medium initially enabled to determine the presence of cellulase in the solution. The cellulose concentration was quantified by measuring the reducing sugar concentrations using the DNSA method [38] [39].

**2.12 Cellulase Activity** [40]

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 oC. The reaction was stopped by the DNSA reagent, followed by heating the mixture to 100 oC. One unit of cellulase activity, one µ mole of glucose released in 30 min from the substrate, denoted as CMC cm-3.

**2.13 Pectinase Activity** [41]

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 0C. The reaction was stopped using a potassium iodide solution, and iodometric titration was performed using 0.2 mM sodium thiosulphate solution and starch as an indicator. One pectinase activity (U cm-3) unit is defined as one µmole of galacturonic acid released from the pectin substrate [42].

**2.14 Protein assay**

The Bradford assay was used to measure the protein content [43].

**2.15 Enzyme Kinetics**

The Michaelis Menten [44] and Lineweaver Burk plots were used to determining the *Km* and *Vmax* values. The Lineweaver Burk plot's slope was equal to the *Km/ Vmax* and the intercept of the Lineweaver Burk plot on the y-axis is 1/ *Vmax*.

**2.16 Spectro- fluorometry**

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 [45].

**2.17 SDS-PAGE**

The extracted enzyme was characterized by performing a reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) [46].

**2.18 Characterization of orange Oil**

**Chemical composition of oil:**

The oil was further characterized using quantitative and qualitative measures including gas chromatography-Flame ionization detector- mass spectrometry (GC-FID-MS) and high-performance liquid chromatography- photodiode array detector- mass spectrometry (HPLC-PDA-MS). Thermo Scientific Co. equipment, BP-1 column was used for Gas chromatography –Mass spectroscopy (GC-MS) analysis with injector and detector temperatures at 240 0C, the heating ramp rate of the oven at 12 0C.min-1 up to 210 0C and at 55 0C cooling temperature. Further, a Thermoelectron corp. Finnigan LCQ advantage Max equipment, with C-18 column, was used for High-performance liquid chromatography-mass spectroscopy (HPLC-MS), using 50: 50 acetonitrile and methanol mixture as mobile phase at a flow rate of 0.3 cm3.min-1.

**Organoleptic Characterization of oil**

The visual assessment of the extracted orange peel oil colour was carried out by comparing it with the standard orange peel oil and the standard limonene. Further the orange peel oil colour was measured using CIELAB color space method [47].

……………..(3)

……………..(4)

The fragrance of the oil was determined by means of sensory analysis.

The density of the oil was determined using Anton Parrs densitometer (Model no. DMA 4500).

The solubility of the oil was checked in water.

The oil specific gravity was determined by following equation

……….(5)

**DPPH Assay (Anti-oxidant activity)**

To understand the anti-oxidant property of the oil the oil was subjected to DDPH assay method with following protocol. The standard DPPH solution has been prepared by dissolving a 24 mg DPPH in about 100 cm3 methanol. The working solution of stock for 0.98±0.02 at 517 nm, 3 cm3 aliquot to 100 μl sample (10- 500 µg. cm3), the mixture was shaked well and kept in the dark for 15 min and after which the absorbance of the mixture was measured at 517 nm.

Further, the oil sample was analysed for its radical scavenging activity using following equation

…….(6)

**2.19 Quantification of orange peel oil**

The yield % of orange peel oil was calculated using:

(7)

1. **Results and Discussion**

**3.1 Selection of suitable colony for orange oil production by SSF**

The oranges peel stored at 8 0C showed the growth of several natural microbial florae 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 1d**).

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**Fig. 1 a** Macroscopic appearance of the different coloured fungal colonies on an orange peel, **b** Macroscopic appearance of selected fungal culture on the orange peels, **c** Orange peel skin separated as layers post fermentation **d** Microscopic image of isolated *Aspergillus sp.* (45 X).

The fungal culture absorbs nutrients from the orange peels and secret cell wall, degrading enzymes that break 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 [19], Torrado et al. [33] and Oberoi et al. [48] have noted the production of enzymes by *SSF* of orange peels using *Aspergillus niger*. However, these reports do not include data on extracting the orange peel oil. Hence, our current work has demonstrated 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 an uncharacteristic colour (**Fig. 2a**) and odour. Agarwal and Bosco [31], Vu et al. [49], Jaiswal and Ghannam [50] have reported that the fungal strains produce pigments that get solubilized 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. 2a**).

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**Fig. 2** The oil obtained by squeezing the peels with (**a)** mixed culture, (**b)** white filamentous growth, (**c)** green growth, (**d)** black growth

**3.2 Selection of orange peel sterilization technique**

Fungi produce pigments that affect the quality of the oil. Therefore, to obtain orange oil that qualifies in the organoleptic test including the bright orange red colour and typical orange fruit flavor, it was necessary to obtain a contamination free fungal growth medium. Thus, sterilization of peels was important [36]. However, there were some challenges associated with each sterilization method, as mentioned below-

**3.3 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. [51] reported extraction of orange oil using steam explosion at high temperature and pressure in less time than hydro-distillation. Yadav et al. [52] reviewed the use of saturated or superheated steam to extract 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 the process energy-intensive. Kannagi and Elangeshwari [53] also conducted fermentation of the steam sterilized orange peels for cellulase production using *Aspergillus* species but have not reported orange oil extraction may be related to the escape of oil from the peels during the autoclaving process. Hence, the steam sterilization of orange peels is not suitable for obtaining fermentation-assisted oil from the peels.

**3.4 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 colour of the 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 airflow in the laminar flow hood. The UV-light treatment still did not prevent microbial contaminations because of the limited penetration of UV-light in the solid peel matrix, leading to *in*complete sterilization of peels [54].

**3.5 Sanitization of peels with 70% ethanol**

The surface sanitization 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 the case of autoclaving and UV-light methods. Gurulingappa et al.[55] have reported the surface sterilization using 70 % ethanol in tissue culture laboratories as an effective method that is in sync with the present research findings.

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 **Fig 3 (a-e)**.

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**Fig. 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

**3.6 Characterization of isolated fungi**

The selected fungal strain showed filamentous growth peculiar to *Aspergillus* strain(**Fig. 4a**) [56]. The colony producing favourable quality orange oil appeared as white growth when seen with naked eyes (**Fig.4 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 (Supplementory Information 1)**.

The isolated *Aspergillus species* possess similar morphological features as that of *Aspergillus niger* [57]. The *Aspergillus species* are generally known to infect food and fruits and cause their spoilage and are rarely regarded as agents to cause opportunistic infections. These are generally recognized as safe (GRAS) category of fungi by the US Food and Drug Administration (FDA) [58]. The *Aspergillus species* consume organic matter at favourable humidity and temperature conditions and are suitable for industrial fermentation processes [59]. The selected *Aspergillus species/*NCIM 1432, grown on an agar plate, showed initial white growth up to 12 h of fermentation period (**Fig. 4 a**), and then formed black spores (**Fig 4 b**).

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**Fig 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

**3.7 Development of seed culture medium**

Puri et al. [60] mentioned that it is essential to optimize seed culture medium and seed culture size based on which the following three mediums were studied to develop seed culture.

**3.7.1 Orange peel as seed culture medium**

The ***Aspergillus species/* NCIM-1432,** when cultivated on an orange peel without any supplementary mediums, showed 26 days of the lag period; hence orange peels as such was not a suitable medium for the culture development. The orange peels used as such can be considered as the control.

**3.7.2 Nutrient Broth as seed culture medium**

When the seed culture was developed in nutrient broth (**Fig 4c**), the culture showed the lag phase for 3 days (**Fig 5**). The seed culture showed an exponential growth on the 3rd day and 4th day, where the fungi showed a loose filamentous pellet structure with about 25-30 number of pellets per 10 cm3 of nutrient broth. The culture attained the stationary phase on 5th -6th day of fermentation, and the growth declined on the 7th day onwards. The short exponential growth and rapid completion of stationary and decline phases in the SmF could be attributed to the agitation during the SmF process, which may break the filamentous fungal strain [61]. In literature, the shear forces created by the agitation during the *SmF* are knownbreak the loose filamentous fungal pellets when the eddy size in the fluid is smaller than the cell size [62].

**3.7.3 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 growing the *Aspergillus species/* NCIM 1432 when compared to nutrient broth medium.

The nutrient agar contains the nutrients that provide nourishment to the microorganisms and enable efficient sub-culturing of the microorganism. The addition of agar solidifies nutrient medium, which makes it suitable for the cultivation of microorganisms and is used for the isolation and preservation of bacterial culture.

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**. The Monod Kinetic plot shows that the specific growth rate was linearly proportional to substrate concentration up to 20 g.L-1 for NA medium and 5 g.L-1 in the 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 (**Online Resource 2**). 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. [61], Janet et al. [63] have reported that *SmF* is not suitable for the growth of fungi. Mariano et al. [42], demonstrated that the shear force during *SmF* damages the filamentous fungi, and only the surface of the fungal pellets are metabolically active. At the same time, low viability zones exist within the pellets. Further, Basu et al. [64], in their literature review and Janet et al.[63] and Mariano et al. [62], in their research studies, have demonstrated that solid medium is the most natural and suited medium for fungal growth which is in sync with the current work.

**Fig. 5** Growth curve of seed culture in **Nutrient Broth:** (Peptone: 0.5%, Beef extract/Yeast Extract: 0.3%, NaCl: 0.5%, Agar: 1.5% - 15gL-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).

**3.8 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 a 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 (**Online resource 3a**).

Thus, the optimum seed culture size was 104 fungi.cm-3, which enabled fungi' mat growth 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. [60], Wu et al. [65], have also projected that optimum seed culture size enhances fermentation efficiency.

**3.9 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 (**Online resource 3b**). It was also observed that on the 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. However, the fermentation beyound 15 days led to browning of the peels, foul odour, and formation of black spores indicating dormant stage or exhaustion of medium and initiation of the 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 (**Online resource 3b**). Thus the 2 mm particle size of the peels showed an 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* speciesto propagate on the orange peels. Torrado et al. [33], Bhargava et al. [66], Zadrazil and Puniya [67] have mentioned that in the case of *SSF* the mass transfer is influenced by the physical characteristics of the substrate, including moisture content, pore size and particle diameter.

The peels of particle size of 0.16 mm showed an extended lag phase, produced a foul odour, and caused the peels' browning indicating the decomposition of peels. Thus, a 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. [66], Torrado et al. [33] and Zadrazil and Puniya [67], have mentioned that unreasonable substrate size reduction leads to a decrease in the water holding capacity of the peels and hence decreases fermentation rate. Hence, it is crucial to optimize the particle size of the substrate for enabling efficient fermentation.

**3.10 Effect of external nitrogen source**

The peels contain only carbohydrates and lack proteins or nitrogen, which are also needed to develop and develop microbial cells [19], [24], [30],[35], [36]. The same issue was faced in the present study owing to which an external nitrogen source was added. In a *SmF* study involving the 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 fermentation rate (**Online Resource 3c**). The lag phase was only 2 days and the seed culture showed matted growth on the 5th day of fermentation. In a *SmF* with orange peel extract, conducted by Rangarajan et al. [68], peptone and soybean meal were used 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 and fermentation rate. Torrado et al. [33] and Adebare et al. [69] had mentioned that nitrogen in the growth medium has a crucial role in microbial growth and enzyme production.

**3.11 Effect of temperature**

The fermentation of orange peel at 25, 30 and 37 ºC in Petri plate produced brown coloured orange peels and the peels lost their freshness in 16 h. Further, the peels appeared dried and stale**.** 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 0C retained the peels' freshness and colour. The oil recovered too was orange in colour.

Hence, the temperature of 8 0C was selected as the optimum temperature. Dhankher and Chauhan [70] have reported that change in the fermentation temperature has minimal or no impact on the product quality, which contradicts the present research findings where a substantial change in product quality is seen with the change to higher temperatures. A similar effect of temperature has been reported in other products such as beer volatiles in the research work demonstrated by Kucharczyk and Tuszynski [71].

Depending on the fungal *species* used (*Aspergillus* species including *Penicillium* atrovenetum, *A.* flavus, *A.* oryzae, *A.* niger*, A.*tubingensis*)* for the 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 [33], [53], [67], [68], [69], [72], [73]. 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 8oC.

However, this temperature (8 0C) was not optimum in the case of enzyme activity*.* The enzyme activity at 8 oC temperature was determined to be 135 ± 0.3 CMC.g-1 and 88 ± 0.2 IU.g-1 for cellulase and pectinase, respectively. The respective enzyme activity at 25- 30 ̊C is 223 ± 0.1 CMCg-1 and 126 ± 0.05 IU g-1 of cellulase and pectinase. The activities dropped at 50 ̊C, to 65 ± 0.08 IU.g-1 (**Fig 6a**) and 121 ± 0.06 CMC.g-1 **(Fig.6b)**. The enzyme recovered by the *SSF* of orange peels showed the maximum activity at 30 0C.

**Fig. 6a** Effect of Temperature (̊0C) on Pectinase activity.

**Fig. 6b:** Effect of Temperature (̊0C) on Cellulase activity.

**3.12 Extraction of fermentation products and their characterization**

**3.12.1 Enzyme Activity**

The oil extraction 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 fermented products' complete recovery, the fermented mass was washed with pH 5.5 citrate buffer solutions. Before moving on to removing cellulase and pectinase enzymes from the fermented mass, it is important to detect these by-products. Hence a zone of inhibition was determined on Agar medium supplemented with cellulose ~~a cellulose agar plate~~ to confirm cellulase activity [39] **(Online Resource 4).**

Once the presence of cellulose and pectinase enzymes was detected, 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 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 iodometric assay [22, 54- 55]. The solution showed the cellulase activity of 11.9 ±0.03 CMCg-1 and pectinase activity of 8.5 ±0.06 IU.g-1, respectively, if plain water was used. While the activity in 50 cm3 citrate buffer of pH 5 was 223 ±0.09 CMC.g-1 of cellulase and 126 ±0.03 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 **(Online Resource 5)**.

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 changes the active sites in the enzyme, thus altering its activities [74], [75]. The studies by Qasim et al.[76], Gangwar & Karthikeyan [77]; Dhembare et al. [78]; show the optimum pH values 4 to 6 for hydrolytic enzymes extracted from *Aspergillus* species*,* [76]*,* [78][79]*.* The optimum pH value for enzymes extracted from *Aspergillus* is 5.5, which can thus be well within the range of pH 4 to 6.

Rangarajan et al. [68] *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. [69] reported using orange peels for the 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 [53] reported 0.3 IU cm-3and 0.62 IU.cm-3of cellulase enzyme by *SmF* of orange peels using *Aspergillus niger* and *Trichoderma viridae*.

Mrudula and Anitharaj [19] reported maximum pectinase production of 1211.2 U.g-1 by *SSF* of orange peels using *Aspergillus niger*. Sajith et al. [73] conducted a detailed literature review on cellulase enzyme production by *SmF* using different fungal strains and found that the enzyme activities 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.

Thus, 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 cellulose) 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 the water while in citrate buffer, 126 IUg-1 (**Fig 7a**) and 223 CMCg-1 (**Fig 7b**) of cellulase of pectinase) falling in the ranges reported by other researchers.

**Fig.7a:** Pectinase 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

**Fig.7b:** Pectinase 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

**3.12.2 Enzyme Kinetics**

The enzyme kinetics plays an important role in analyzing the enzyme efficiency. The *Vmax* value of the cellulase enzyme was 7.31 x 10-5 mg /cm3 /s, and its *Km* value was 6.3 mg/.cm3. The *Vmax* value of the extracted pectinase enzyme was 3.2 x 10-4 mg /cm3/ s, and its Km value was 3.8 mg (**Figure 8 a-b**). The reported *Km* value for pectinase, in the existing literature, is in the range of 0.0201 – 4.22 mg/cm3 and the *Vmax* values for the pectinase are reported as 6.6 x 10-4 mole/cm3/s[80]. The reported *Vmax* values for the cellulase are 2.89 x 10-4 mole/cm3/s to 6.94 x 10-4 mg.cm-3.s-1 and the values for *Km* is 0.28 % CMC [81]. The Km determine the affinity to the substrate and the *Vmax* values indicate the kinetic efficiency of the enzyme. The enzymes generated in the *SSF* process indicated moderate affinity and a moderate rate of reaction. Such behaviour can be because of the inhibitory effects of other fermentation products such as sugars.

**Fig 8a:** Michaelis Menten representation: The plot of glucose released v/s substrate concentration, □-Cellulose, ◇- Pectin, S: Substrate Concentration.

**Fig. 8b: Lineweaver Burk representation:** Plot of the inverse of glucose release per unit time v/s inverse of substrate concentration

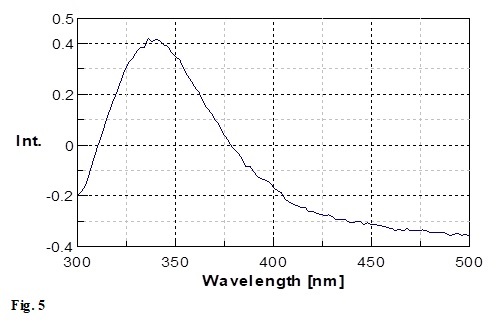
**3.12.3 Enzyme Purification and Recovery**

The enzyme purification at 0- 4 oC prevented enzyme denaturation. The ammonium sulphate precipitation produced enzyme activities as given in (**Online Resource 5**). 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.

**3.12.5 Spectro-fluorometry**

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 a prominent fluorescence peak at 342 nm (**Fig. 9**). The active sites of the cellulase consist of amino acids, including tryptophan. The indole ring of tryptophan possesses an internal fluorescence activity [25].

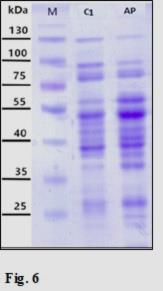


**Fig. 9** Emission Spectrum of enzyme extract after ammonium sulphate precipitation

**3.12.6 SDS-PAGE Chromatography**

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. 10)**.

The molecular weights for the extracted enzymes are given in **Fig. 10**. 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. viridae*has shown molecular weight close to 39.2 KDa [7]. In the present research, the molecular weight, as determined by reducing SDS-PAGE, is around 30 and 55 kDa, which is almost identical to molecular weight well-characterized by Pirzadah et al., [62] and Ahmed et al., [7].



55-58 KDa Band

36-40 KDa Band

**Fig. 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

**3.13** **Characterization of extracted orange peel oil**

The HPLC-MS showed Tangeritin, Sinensetin, Nobiletin, Tocotrienol, and Tocopherol acetate in the oil. The oil composition is comparable with different processes in literature [82], [83], [84] (**Online Resource 6**). The GC-MS analysis of the oil showed α-pinene, d-limonene, limonene oxide, linalool and citronellal in the extracted oil (**Online Resource7**). The composition of the oil is comparable to the composition of oil obtained by the supercritical extraction process [9].

The visual assestment of the extracted orange peel oil revealed the color of the extracted oil had 56.8, 4.7 and 12.2 L\*a\*and b\* values respectively hence the oil was bright orange in color. The oil had typical odour of a fresh orange fruit. The density of the oil was 0.86 kg.m-3. The oil had specific gravity value of 0.87. The oil was hence lighter than the water and was floating above the water in the reaction mixture.The oil had antioxidant property as analysed by DPPH assay method (**Section 2.17, Online Resource 8**).

**4 Optimized conditions**

The extraction of orange peel oil was conducted by treatment with *in-situ* generated extracellular hydrolytic enzymes secreted by the isolated fungal colony using *SSF* technology. Owing to the utilization of the by-product of fermentation i.e. the extracellular enzymes for the extraction of oil, the method can be termed a green method. The fermentation parameters included in the process of extraction are sterilization technique (70 % v/v alcohol), fermentation temperature (8 oC), external nitrogen source (2% w/v tryptone), an average orange peel particle size (2 mm), and inoculum size (104 fungi cm-3). During the 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 0C extraction temperature range.

**5 Conclusion**

A green, sustainable alternative to the available orange peel oil extraction methods has been developed to extract orange oil. The method enables the production of enzymes as a by-product during the process. The developed process has used low tech equipments. The developed fermentation method needs a longer time for producing the enzymes and in turn the oil, compared available orange peel oil extractions processes in literature, which is the major limitation of the current process. Hence the developed process will be suitable mainly for manual production of orange peel oil and needs further modifications for making the process suitable at large scale. However, the developed process is beyound only enzyme production by fermentation and hence has definitely opened a new area for orange peel oil extraction.

**Abbreviations**

|  |  |
| --- | --- |
| BLAST | Basic Local Alignment Search Tool |
| CMC | Carboxy methylcellulose |
| 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 |

**Declaration:** This work is an original piece of work. The work has a simultaneous pre-print copy available online with Research square team. Following is the link of the pre-print <http://scholar.google.co.in/scholar_url?url=https://www.researchsquare.com/article/rs-123470/latest.pdf&hl=en&sa=X&d=11037927583404371992&ei=ggfhX522I8aOmgGjlJPABQ&scisig=AAGBfm09kwM_Leh23hM4cLDkk5fUUpBH0w&nossl=1&oi=scholaralrt&hist=MnxfrD0AAAAJ:8986658008029181119:AAGBfm2HD_b0e9qD4-vjNbI3X5KjrhtMaw&html=> The research square team has claimed that the work qualifies to be an original piece of work.

**Consent for publication:** YPL is willing to publish the work

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

**Code availability: No code was used**

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**Details of the AI usage are given below:**

**1.**

**2.**

**3.**

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