Valorisation of citrus waste for the extraction and characterization of limonene

Major Project Report

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DECLARATION

We hereby declare that project on "**Valorisation of citrus waste for the extraction and characterization of limonene**" has been done by us under the supervision of **Dr. ASHOK KUMAR NADDA Assistant Prof, Department of Biotechnology and Bioinformatics.** Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India. We also declare that neither this project nor any portion of this project has been submitted for the granting of any degree or diploma elsewhere.

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CERTIFICATE

This is to certify that the work presented in the project report titled **" Valorisation of citrus waste for the extraction and characterization of limonene**" in partial fulfillment of the requirements for the award of the degree of B.Tech in Biotechnology and submitted to the Department of Biotechnology And Bioinformatics, Jaypee University of Information Technology, Waknaghat is an authentic record of work carried out by "**Mr. CHAITANYA MOGHE (181819**)" during the period from August 2021 to May 2022 under the supervision of **Dr. ASHOK KUMAR NADDA**, Department of Biotechnology And Bioinformatics, Jaypee University of Information Technology, Waknaghat Solan Himachal Pradesh India.

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To the best of my knowledge, the preceding statement is correct.

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ABSTRACT

Citrus waste is a growing concern in the modern-day world, due to poor waste management and lack of knowledge which has proven to be harmful to the soil when used in landfills thus emitting greenhouse gases (CO_2 and CH_4) resulting in global warming and burning which results in the release of dioxins These fruit wastes can be utilized as low-cost biomass in the production of value-added products like Cellulase, pectinase, protease, lipase and amylase enzymes and help in the production of biofuels and food industry, further lowering the cost of production of enzymes.

The complete utilization of the citrus waste into value-added products provides an alternate pathway to achieve commercially valuable assets like limonene, cellulase, amylase, and the use of orange processed powder as an alternate carbon source in media preparation.

The limonene production from the soxhlet approach was not that convenient as the quality of limonene was not very effective in demonstrating the zone of inhibition properly on culture plates by well diffusion technique. Although the colour imparted by the sample was dark orange with a hint of an orange smell in it. In spite, of the quality of limonene being affected, the processed orange powder by soxhlet was found to be a competitive carbon source against beef extract.

Keywords: Orange peels, Limonene, Citrus waste, Anti-microbial, Media preparation, Cellulase, Amylase

CHAPTER - 1

INTRODUCTION

The world population is increasing gradually so is the demand for food resources. Fruit waste is one of the major concerns in fruit based waste management as a huge percentage of the whole fruit is turning into waste using as landfills. Citrus Fruits are consisting of nearly 50-60% of peel, which is considered and discarded as waste. In many cases it has been seen that the waste content is greater than the product itself *(Wadhwa and Bakshi 2013).* Utilizing even the waste may result in greater potential in the production of various commercially beneficial enzymes and natural antimicrobials like limonene. The utilization of citrus waste is economically benefitting industry and ecologically benefitting the world. The orange peels were found to be composed of water-soluble materials like carbohydrate (glucose, fructose, cellulose, pectin, hemicellulose) proteins, crude fat, bio-oils, and bio-active compounds like limonene. Due to limonene's antimicrobial activity, the citrus waste is hard to degrade by the natural microbes present in the environment preventing its conversion into bio-decomposable manure and for the production of commercially beneficial enzymes. But itself being a commercially valuable product, limonene production can further benefit the industry. The integral valorization of food waste and utilization of high content value-added bioactive molecules as novel raw materials can possess several bioactive applications. *(G´omez-García et al., 2021b).*Use of by-product valorization results in the production of different bio-products which further helps in improving different sectors, like production of phytochemicals, enzymes, functional ingredients, biofuels *(Ravindran and Jaiswal,2016).* Methane is produced by lime peels through anaerobic digestion *(Lane, 1983), Long and Patrick (1965)*was successful inyielding (2.3 to 4.4%) of 2,3-butylene glycol by *Bacillus polymyxa* by converting orange by-products via the fermentation process. Citrus waste was utilized by *Garzon and Hours (1992)* for production of pectinase using *Aspergillus foetidus(NRRL 341, ATCC 16878). Chedea et al. (2010***),** performed an experiment on orange peels and extracted carotenoids by acetone and hexane based extraction. The carotenoids extracted were10'-apo- β- carotene-10'-ol, 10'- apo- β-caroten-10'- al, 8'-apo-β-caroten-8'-al, and 6'-apo-β-caroten-6'-ol.Due to low nitrogen content *(Mahato et al., 2019)* and

detrimental nature towards soil microbes due to the presence of antimicrobial activities citrus peel wastes are not suited for composites*(Zema et al., 2018a).(Forgacs et al., 2011)*extracted limonene by the steam explosion method from orange peel, same technique was also performed by *Pourbafrani et al. (2010)* on orange peels as a substrate for the production of limonene and stated 94.3% productivity of limonene. An alternate method was used by *Wikandari et al. (2013)* for the extraction of D-limonene via solid-liquid extraction/ n-hexane methods and succeeded to achieve 80% productivity.

The complete utilization of the orange peels can result in the production of many commercially viable products and better waste management processes. But due to limonene's antimicrobial activity, the citrus waste is hard to degrade by the natural microbes present in the environment preventing its conversion into bio-decomposable manure or commercially utilizing waste for the production of commercially beneficial enzymes. By extracting limonene from the orange peels, the resulting peels can support microbial growth as a good carbon source. In addition, limonene is a commercially valuable compound and can be used in fragrance, flavouring, solvent, preservatives, cleaning, industry, etc. Limonene can also be used as a precursor for making various compounds like menthol, poly-limonene, limonene epoxide polymers, etc. After the extraction of limonene, the resulting orange peel powder can be utilized as a carbon source in media components for culturing microbes. As the orange powder is rich in components like cellulose, hemicellulose, fats, and pectin. We can utilize the powder for manufacturing various enzymes like cellulases, amylases, pectinase, lipase, etc. but after experimentation, it's clear to create optimal strategies for better extraction of limonene for forming concentrations which clearly shows the anti-microbial activity comparable to anti-biotic competitors.

On the contrary, various studies conducted by many researchers have proven that orange peels can be a good substrate for the production of enzymes as it is rich in monomers and polymers of carbohydrates and proteins. A study conducted by *Mamma et al. (2008)*found that orange peel composition has 41.1% water-soluble materials (carbohydrates: glucose: 14.6%; fructose 15.5%, 16.2% cellulose, 14.4% pectin, 13.8% hemicellulose, 7.9% protein, 3.9% crude fat and 1.7 % ash). Theafore mentioned composition makes orange peels a suitable candidate as a substrate for the production of various enzymes through microbe.

Use of by-product valorization results in the production of different bio-products which further helps in improving different sectors, like production of phytochemicals,

enzymes, functional ingredients, biofuels *(Ravindran and Jaiswal,2016).* Methane is produced by lime peels through anaerobic digestion *(Lane, 1983), Long and Patrick (1965)*was successful inyielding (2.3 to 4.4%) of 2,3-butylene glycol by *Bacillus polymyxa* by converting orange by-products via the fermentation process. Citrus waste was utilized by *Garzon and Hours (1992)* for production of pectinase using *Aspergillus foetidus(NRRL 341, ATCC 16878). Chedea et al. (2010***),** performed an experiment on orange peels and extracted carotenoids by acetone and hexane based extraction. The carotenoids extracted were10'-apo-β- carotene-10'-ol, 10'- apo- β-caroten-10'- al, 8'-apo-β-caroten-8'-al, and 6'-apo-β-caroten-6'-ol.Due to low nitrogen content *(Mahato et al., 2019)* and detrimental nature towards soil microbes due to the presence of antimicrobial activities citrus peel wastes are not suited for composites*(Zema et al., 2018a).*

Soxhlet method was use to extract limonene present in oil from the orange peels using hexane as a solvent. In by-product processed and dried orange peel powder was obtained which doesn't contain limonene or any volatile compounds. We can utilize the powder as an alternate carbon source instead of beef extract to grow microbial culture for manufacturing various enzymes like cellulases, amylases, pectinase, lipase, etc.

After experimentation, it's clear to create optimal strategiesfor better extraction of active limonene in forming effective concentrations which clearly shows the anti-microbial activity and create definite zone of inhibitions which can be comparable to its anti-biotic competitors.

CHAPTER – 2

LITERATURE REVIEW

Analysis and utilization of orange peels as a substrate

Citrus fruit peels are rich in fermentable sugars and low in lignin, their vast abundance makes them desirable low-cost source for the production of biofuels and enzymes *(Madhavan et al., 2016).*Study conducted by*Mamma et al.(2008)*found that orange peel composition has: 41.1% water-soluble materials (carbohydrates: glucose: 14.6%; fructose 15.5%, 16.2% cellulose, 14.4% pectin, 13.8% hemicellulose, 7.9% protein, 3.9% crude fat and 1.7 % ash. Furthermore, *Balu et al.(2012)* used the microwave for obtaining four fractions from orange peel (sugars, cellulose, pectin, bio-oil). Orange peels were treated in the microwave and filtered afterward to obtain cellulose (9%, DW) in solid form. The resulting filtrate was cleansed by ethanol to recover pectin (8%, DW). From liquid-liquid extraction with ethyl acetate, bio-oils (7%, DW) were extracted wherein total solvents were collected and then evaporated to collect sugars (26%, DW). They concluded that around 26 % of the dry weight is sugar and 9 % is sugar (carbohydrate) derivative. Thus, yeast can convert sugar into alcohol and CO_2 whereas glucose is expected to produce more CO_2 than any other sugar substrate.*(Cekmecelioglu,.et.al (2013)).* The carbohydrate utilization was observed in range from 2.24 on day 20 and declined to 0.17 on day 90 by fermentation *(Cekmecelioglu,D.et.al (2013)).* The molasses utilization can be observed as an indication of utilization of carbon sources, the range initiates from 2.12 in 1st week and declined to 0.07 by the 12th week. The molasses utilization by yeast metabolism results in the production of sulfur dioxide (*S kariyachan et.al (2015). The* protein content got declined from 2.18 in 1st week to 0.30 in the 12th week. Banana and Orange show higher protein content (1.2) due to indigestion of the cellulosic wall.

Yeast transforms lemon sugars (carbohydrates) into ethanol during fermentation (4- 5 w/v %). Temperature and O_2 concentration play an important role in optimization of product. Further, the ethanol gets converted into acetic acid, the total acetic acid content from nil at day 1 to 1.7 till day 90*(Daneshzadeh Tabrizi et.al, 2010).*Orange peel waste was collected by *(Oussadi et.al (2015))* from juice shops and were analyzed for chemical composition: the

results demonstrated as- total sugars %(carbohydrates) = 44.5 ± 1.65, protein % (crude) =3.71 ± 0.05, fat% (crude) = 0.39 ± 0.001 and Ash % = 3.46 ± 0.27. *(Oussadi et.al 2015).*

Boukroufa et al. added microwave and ultrasound in the process and further proposed "in-situ" recycling of orange peel water to obtain pectin (4.8 kg/100 kg), polyphenols (11.7 g/100 kg), and essential oil (346 g/100 kg). This biorefinery achieved a reduction in-time of process, energy and waste-water generation. *(Boukroufa et.al (2015)).*

An experiment performed by *(Aartheeswari. S and Dr. B. Kirthiga (2021))* found that lower pH (due to the presence of acidic amino acids) increases the fermentation rate, as it affects the shape of the proteins (enzyme) which helps in fermentation reaction. The pH of the reaction continues to drop due to the formation of organic acids from ammonium ions by yeast cells. During the experiment, the weight of fruit peel waste was measured and 10 ml of yeast *(Saccharomyces spp.)* culture and 5 ml of *Bacillus subtilis* MTCC 2274 were added and mixed with 300g of citrus fruit peel and 100g of molasses within 1000ml of water in the bottle. The container was vigorously shaken to dissolve sugar; the process gets repeated at least two times a day for a span of 2 months at 35 ˚C for fermentation. The sample solution takes 90 days to fully ferment. The fermented solution gets analyzed every week for biochemical activity change. By taking a 5 ml sample of bio cleaner solution the pH gets measured by a pH meter. The initial pH of the bio-cleaning sample for the first week was found to be around 4.23, a gradual declination in the pH was demonstrated by the 12^{th} week to 3.10. citric acid pH was compared with banana (2.40), watermelon (2.45), orange (2.46) grapes (2.48), lemon (2.50), and apple (2.53). storage conditions do affect the pH as the acid oxidation results in higher pH.

BIOREFINERY APPROACH

Bio-refineries can be defined as a sustainable and integrated approach ofthe conversion of biomass into various materials and chemicals which can be used as fuels to generate power *(Cherubini, et al., 2007).* Hybridization of various technologies can be seen in various fields including microbiology, chemistry, and engineering which gets incorporated and molded in a process to disintegrate biomass into building blocks, such as proteins, carbohydrates and lipids, and oil. Further, these building blocks gets converted into value-added products such as biofuels or energy-generating chemicals*(Cherubini, 2010).*

Bio-refinery processing of food waste can produce multiple value-added products and has significant advantages over conventional technologies including optimized utilization of

feedstock, covering multiple markets for diversification of revenue, minimization of waste production and processing, different technologies working in synergy by sharing manpower and machines, self-efficiency potential by biogas production.*FitzPatrick, M et.al (2010).*

Solid-state fermentation(SSF) is the most promising approach to reduce production and purification costs for the enzyme and outclassing advantages over the submerged systems for coproduction of enzymes by simple equipment requirement, high enzyme production rate with a reduction in medium and overall cost, resembling the natural environmental conditions. *(Wolski E et,al (2009))* Pectin can be directly hydrolysed in order to reduce purification cost in solid fermentation.

Solid previously used in the production of biodiesel was fermented *(Soares D et. al (2013))* and these fermented solids were utilized as precursors at a commercial scale to deduct the process and purification cost. By using non-sterilized fermenters and using fermented solids D –galacturonic acid by pectin using pectinase was produced *(Biz A et.al (2016)).*The initiation of the process begins with washing out contaminants from the substrate followed by drying at 105 ˚C for 6 hours in the oven after, 12 hours of exposure to sun. the substrate was mechanically grinded into 0.42 mm particle size.

For protease production *Penicillium expansum* CMI 39671 strain was used *(Bernfeld P et.al (1955)).* The strain was exposed to UV for mutation and mutants were analyzed for lipase and pectinase production. As substrate, fruit waste (orange peel, lemon peel, papaya peel, apple pomace, and banana peel) was used along with peptone, yeast extract, beef extract, casein, ammonium sulfate potassium nitrate, sodium nitrate, and corn steep liquor as nitrogen sources.Organic nitrogen sources enhance microbial growth and enzyme production. *Qureshi AS et.al (2016)).* Although the fruit waste was rich in proteins additional utilization of nitrogen sources enhances the enzyme production. Both the enzymes (lipase and pectinase) increased production with temperature up to 40˚C which presents the optimum temperature as increasing temperature after this mark results in decreasing production.

In the time frame for orange peel as a substrate, the concentration of pectinase and lipase increased till 5^{th} day after that the production rate started dropping. The reason could be decreasing pH due to organic acid production, as pH plays an important role in enzyme production.

In SSF moisture is vital as well, the results indicated that the moisture content before fermentation can be a critical factor with 70% being optimal in the case of the production of

the enzyme via SSF. An alteration in optimal moisture value may result in a change in physical properties thus affecting the production of enzymes as well. The higher value of moisture content can be denied as the porosity of the solid substrate gets decreased by increasing the moisture content of the substrate resulting in poor gas exchange.Fruit peels are a rich source of phenolic compounds, hemicellulose, phenol, and terpenic compounds which consistof 15- 50% of total fruit *(Pathak et al.2015, 2017).*Advancement in process intensification technologies strategies like ultrasound and microwave-assisted steps provides an alternative to conventional methods.

Bio-refinery can achieve better economic efficiency by combining products of high-value low-volume products (e.g. phenolic compounds, essential oils, pectin, etc.) and low-value high-volume products (e.g. cattle feed, compost, etc.). The general process used includes size reduction of fruit peel waste followed by the drying process. Phenolic compounds got extracted by the process of solvent extraction. *Cherubini, F.et.al (2007).* Phenols can be used as antioxidants. Essential oils can be separated by the process of steam distillation. By adding acid Non- reducing sugars get converted to reducing sugar through the process of hydrolysis (*FitzPatrick, M et.al (2010)).* Hydrolysis is essential for attaining higher yields of fermented products. Syngas can be obtained by the solids separated through the process of hydrolysate filtration. The liquid phase of hydrolysate is further processed to get biochemical extraction or it can be used for fermentation. The fermented products through regular distillation can produce ethanol in purified form.*(Cherubini, F.et.al (2007))*

Through the bio-refinery approach Fruit peel wastage can be further processed into making bio-energy [direct burning, Liquid ethanol, methanol (pyrolysis of fruit peel waste)], bio-chemical extraction (proteins, enzymes, aromatic compounds, phenolic compounds, etc.), and obtaining bio-materials (adhesive, dyes, activated carbon, nano-materials, antimicrobial edible films etc.).Even though the raw materials are bio-degradable the conversion process is not environmentally inert. Thus, evaluation of the environmental loading is to be considered to make the process and design sustainable.

PRODUCTION OF DIFFERENT ENZYMES BY CITRUS FRUIT PEEL AS SUBSTRATE

*Abdul Sattar Qureshi et.al (2017)*used *Penicillium expansum* CMI 39671 (thermophilic fungal strain) as a fermentation strain for coproduction of pectinases and lipases using several fruit waste and compared their production in sterilized and non-sterilized conditions. Lipase

and pectinase's highest activity (1870 U/g and 2817 U/g dry substrate respectively) was shown from orange peels feedstock with no significant difference when compared between sterilized and unsterilized conditions and the lowest activity was found in banana peel feedstock (1266 U/g for lipases and 1662 U/g for pectinases) when compared sterilized versus non-sterilized process pectinases and lipase enzyme demonstrated (13791 U/g) and (8114 U/g) respectively in sterilized conditions and (14091 U/g) and (8324 U/g) respectively in non-sterilized conditions. Non-sterilized was demonstrated with an inclination in enzyme production when compared to sterilized conditions. According to Maillard's reaction, sterilization may result in decreasing the nutritional value of the fermented medium *(Martins et.al (2000)).* Irrespective of similar trends followed by both of the enzymes, pectinase showed higher enzymatic activity over lipases. For optimum process conditions, peptone was used as a nitrogen source at pH 7 and a temperature of 40 ˚ C for 5 days. In addition, bacteriocin-like compounds were produced by the fungal strain resulting in the inhibition of microbial growth *(Raimbault M et.al (1998)).* This study may further help in developing a process that is cost-effective, less energy-intensive, and robust. Using open non-sterilized fermenters SSF for fruit waste can favor cost reduction for lipase and pectinase production.

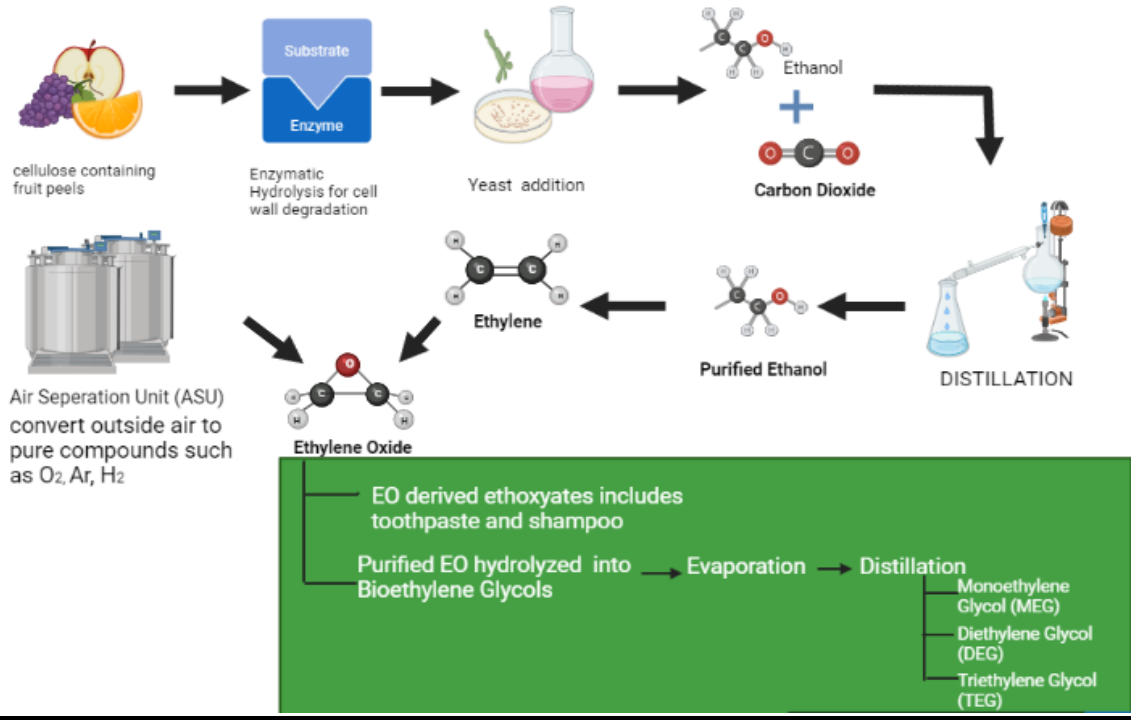


Figure 1: Utilization of orange peels on an industrial scale

CELLULASE

Cellulases is a group of enzymes that includes β-glucosidases (*β-glucosideglucohydrolase; cellobias*e), endoglucanase (*1,4-β-D-glucan-4-glucanohydrolase*), and exoglucanase (*cellobiohydrolase; 1,4-β-D-glucancello-biohydrolase*). Cellulase can be used in the paper and pulp industry, textile, food and beverages, waste management, biofuels and pharmaceutical industries. *(Srivastava et al., 2021*). Their functionality is to depolymerization of cellulosic biomass and convert it into fermentable sugars via hydrolysis process. *(Kuhad et al., 2016).* Due to the presence of hemicellulose, pectin, lignin, and minerals hydrolysis of cellulose is difficult, thus pre-treatment of the substrate is required for improving the yield of fermented sugars. *(Kuhad et al., 2016).*

Till now, cellulase demand is being fulfilled by the submerged state fermenters (SmF)**(Suksong et al., 2019).,** However, the cost of production through this process is cost-intensive due to maintaining strict aseptic conditions, excess water, low yield and the high cost of the down-streaming process. The cost of production via SmF process is approx. (20 $/kg). Solid-state fermenters (SSF) is proven to be an effective alternate, as it facilitates ease of access, less consumption of resources due to utilization as a solid substrate, less waste water production, simple fermentation medium, less capital cost, and higher productivity**.** *((Patel et al.,2017)).* The cost of production via the SSF process was approx.(0.2 $/kg)*(Srivastava et al., 2021)).* the cost of production of cellulose via SSF is ten times more cost-efficient than current SmF production. *(Srivastava et al., 2021)).* The barriers to the process advancement are, incomplete mass and heat transfer, ineffective design, and unavailability of the low-cost substrate *(Caldeira et al., 2020).*Component of cellulose along with sugar availability in the waste seems to be a factor influencing improvement in cellulase's production forlong-term feasibility at a large scale. *(Srivastava et al., 2021).*

Cellulase is secreted by fungi like actinomycetes. These secreting microbes are classified by their complexity (complex and non-complex), respiration nature (aerobic and anaerobic), optimum temperature conditions (thermophilic or mesophilic***).****(Kuhad et al., 2016; Haldar et al., 2016).*Filamentous fungi like *Trichoderma species* (*T viride, T. reesei*, and *T. longibrachiatum*) are known to break down lignin and can be utilize to break ligno-cellulosic biomass by hydrolysis for fruit substrates (orange, and other citrus species) *(Bischof et al., 2016).* Mutant *T. reesei* can produce at least 2 *Cellobiohydrolase* (CBHs), 7

Beta-Glucosidase (BGLs), and 8 Endoglucanases (EGs) and can be profitably transformed into cellulase production.

Irshad et al. (2013), reported *Trichoderma viride*for the production of cellulase enzyme using orange peels in SSF fermenter under optimal conditions. Maximum recorded production of β-glucosidase (515 ± 3.7 U/mL), exo-glucanase (412 ± 4.3 U/mL), and endo-glucanase (655 ± 5.5 U/mL). *Li et al. (2015),* pre-treated microwaved orange peel powder with surfactant PEG 4000 and with the help of *Aspergillus japonicus* PJ01 demonstrated higher-level (more) production of enzymes including cellulase, pectinase, and xylanase via SmF. *Anuradha et al. (2010)* reported multi-enzyme production of enzymes (like xylanase, cellulase, amylase, protease, and polygalacturonase using*Aspergillus awamori* MTCC 9166 and cellulosic orange peel as substrate.*Srivastava et al. (2017)* used orange peels as a substrate (concentration -6.0g) with microorganisms *Emericella variecolor* NS3 at 50 ˚ C and pH 5. The cellulase activity was reported 31 IU/gds FP. *Santi et al. (2015)* conducted a study with an extended approach towardthe production of bioethanol from orange peel waste by a series of processes like acid-catalysis, steam explosion, enzymatic saccharification by enzyme treatment using pectinase and cellulase The resulting solution was further reduced using *Saccharomyces cerevisiae* F15.

Cellulase contributes significantly to the development of biofuels, by diversifying the application of cellulase which results in improving their efficiency.*(Srivastava et al., 2015).* The current scenario is being obstructed by the incomplete efficiency and high production cost. High cost of commercial cellulase is a major impacting factor in higher cost of biofuels when compared to existing transportation fuels *(Srivastava et al. (2021)*

The combined action of the cellulase cocktail (endoglucanase, and exoglucanase) converts the pre-treated cellulose into oligosaccharides and further*β-glucosidases* convert the oligo-saccharides into monomeric reducing sugars. Then via fermentation process these monomeric units to be converted into different biofuels with the use of different microbes. *(Srivastava et al., 2021).*For bioethanol production*Singh et al. (2015),*conducted a study with orange peels as substrate, he treated the substrate initially autoclaved and then with cellulase enzymatic hydrolysis activity, furthermore, fermented the monomeric sugars using *S. cerevisiae* (yeast) with SSF, the bioethanol maximum yield observed was [4.07% (v/v)] at 40˚ C. the maximum incubation time and pH for orange was observed at 48 hrs at 4.0 pH by

*Corbin et al. (2015).*High solid orange peel waste loading in diluted acid hydrolysis results in improved ethanol production.

In present days, Species /Strain used by different Companies like Novozyme use (*Aspergillus niger, Trichoderma reesei* (ATCC26921), Yakult (*Trichoderma viride*) and DuPont (*Trichoderma reesei)* for commercial production of cellulase.

AMYLASE

Amylase is a crucially required industrial enzyme and represents approximately 25% of the total enzyme business worldwide because of their wide application in baking, brewery, food, detergent, textile, and paper industry *((Li et al. 2011) (Agger et al. 2001).* It can be extracted from microbes, plants, and animals. Microbially produced α-amylase has vast industrial applications due to higher performing stability, economic bulk production capacity ease to manipulation *(Lonsane and Ramesh 1990),* cheap cost than animal or plant produced amylase.*(Grupta et al. 2003)*

Maximizing enzyme production by optimization of cultural conditions (agitation, temperature, pH, dissolved oxygen, inoculum size) is a desirable goal in the biotechnology processes/industry. The use of synthetic media in the production of *α -amylase* is very uneconomical and expensive. To reduce the cost of production the agro-industry-based substrate must be utilized *(Balkan and Ertan 2007). (Rivas et al. 2008; Balu et al. 2012)*stated orange peel as a low-cost potential source. Orange peel waste contains 42.5 % of pectin, 16.9 % soluble sugars, 10.5% hemicellulose, and 9.21% cellulose**,***(Rivas et al. 2008).*The SmF is preferred because of its ability of greater control over factors like temperature and pH, easy handling, it has taken over around 90% of the enzyme production for industrial applications. Optimization of a culture media component is a critical task as it influences microbial growth and enzyme production. Optimization of cultural parameters includes inoculum density, carbon, and nitrogen sources, and physiological components like pH and temperature. *(Djekrif-Dakhmouche et al. 2006; Cotârlet 2013).*

The agro-industrial orange fruit waste is rich source and provides all the required nutrients for microbial and enzymatic growth. *(Shahera et al. 2002)* stated that orange peel was proved to be the best inducer for the *α -amylase* growth.*Demir et al. (2012) and Embaby et al. (2014)* reported the production of polygalacturonase from *Aspergillus sojae M3* and *Bacillus licheniformis* SHG10 respectively. *(Singh et al. 2011)* quoted that the *D-sorbitol* and

D-inositol were the best inducers for *α -amylase* production followed by the starch as the presence of starch was inductive as an enzyme-substrate for the production of *α-amylase(Ray 2001).*

Orange peel waste was collected from juice shops were analyzed for chemical composition: the results demonstrated as- total sugars %(carbohydrates) = 44.5 ± 1.65, protein % (crude) =3.71 ± 0.05, fat % (crude) = 0.39 ± 0.001 and Ash % = 3.46 ± 0.27. The waste was first air-dried and then sieved by 0.250 mm diameter apparatus. A suspension was prepared with the help of distilled water of 5% and 10%. vortexed and then centrifuged at 6000 rpm. *(Oussadi et.al (2015)).* The pH range between 5-9 was found to increase the *α-amylase* production significantly by 52.85%, which indicates the alkaline nature of *α-amylase* extracted from strain *Streptomyces sp.* (20r) and have an optimal pH at 9 *(Syed et al. (2009))*and *(Oussadi et.al (2015)). (Kondepudi and Chandra 2008)* quoted the tremendous potential of alkaline *α-amylase* in the detergent industry. For the growth of haloalkophile microorganisms, a high salt concentration is required along with the presence of alkaline pH. In *(Oussadi et.al (2015))* research, the addition of 6.5% (1.11 M) of *NaCl* increases the enzyme production by 45.31%. These halophilic conditions may inhibit the growth of many undesired organisms and enzymatic conversions (Ventosa and Nieto 1995). Most of the *α -amylase* is known to be calcium (Ca^{2+}) metalloenzymes. The calcium ion is required to maintain spatial confirmations. However, the *Streptomyces strain 20r* extracted enzyme seems to be independent of Ca^{2+}ions, as it doesn't show any changes in performance when Ca^{2+} ion is added in the media.Inoculum size is the most important factor, affecting *α -amylase* release. It positively affects enzyme production by 29.42%. in *Nocardiopsis aegyptia. (Abou-Elela et al. 2009).* The same outcome was presented by *(Kammoun et al.2008)* with *A. oryzae.*The excess nitrogen source also demonstrated the inhibitory activity, which can explain the addition of any other external nitrogen source either depleted the growth or have no change when used with the orange peel as a substrate *(Oussadi et.al (2015)).* This may also be due to the addition of more than one complex nitrogen source. But a contrasting result was demonstrated on *Streptomyces strain A3* by *Chakraborty et al.(2012)*which supported growth by the addition of an external nitrogen source.The key determinants for *α –amylase* production extracted from *Streptomyces sp. (20r)*were determined by Plackett-Burman design and the optimization plan was determined by response surface methodology wherein the optimum levels were determined by central composite design (CCD).

Amylase is a crucially required industrial enzyme and represents approximately 25% of the total enzyme business worldwide because of their wide application in baking, brewery, food, detergent, textile, andpaper industry*((Li et al. 2011) (Agger et al. 2001).*

LIMONENE AND ORGANIC ACID PRODUCTION AND UTILIZATION IN BIOREFINERIES

Citrus fruit waste includes pulps, peels, and seeds. Using citrus waste an integrated process was developed by *Pourbafrani et al* for extracting value-added products like ethanol, pectin D-limonene, and biogas *(Pourbafrani M et.al 2010).* The process of D-limonene (8.9 L/ton) initiates with pre-treatment of citrus waste by using dilute acid under high pressure for hydrolyzing the biomass, followed by explosive pressure reduction resulting in the extraction of Limonene. Furthermore, the pectin (38.8 kg/ton) can be extracted by centrifuging the remaining hydrolysate and obtaining the liquid part of solvent precipitation and ethanol (39.6 L/ton) by fermentation. The remaining stillage after ethanol fermentation can be utilized through anaerobic digestion for biogas production (45 m^3/ton).

With the help of a newly developed RP-HPLC chromatogram different organic acids concentration was analyzed in different citrus fruits and among all ascorbic acid and citric acid was proved to be predominant. The citric acid content was highest in *C.aurantium* juice 35.36 mg mL^{-1} and ranged between 3.40–35.36 mg mL^{-1} in different fruit samples. On the other hand, the highest proportions of ascorbic acid were found in *C. nobilis* (0.54 mg mL^{-1}) and ranged between 0.083 and 0.54 mg mL^{-1}*(Pourbafrani M et.al 2010)*. In a study conducted by *Scherer et al. (2012)*, 67.97 mg mL^{-1} citric acid and 0.29 mg mL^{-1} ascorbic acid in *C. aurantifolia*juice was reported which indicates the importance of the role of environmental and analytical processes.

The pH range for citrus fruit juices is 2.45 to 4.07. The ˚ Brix represents the soluble solid percentage in values for all the citrus samples ranging from 6 ˚ to 13˚. *(Pourbafrani M et.al 2010).*When total carbohydrate (glucose, sucrose, fructose) concentration was observed in different fruit juices source,*C. nobilis* demonstrated the highest carbohydrate content in sucrose equivalent (140 mg mL-1), glucose (537 mg mL-1), and fructose (125 mg mL^{-1}).*(Pourbafrani M et.al 2010).*Steam explosion is applied on lemon peels by *Boluda-Aguilar and López-Gómez (2013)*to get essential oils which were further processed by sequential and simultaneous fermentation and hydrolysed to obtain ethanol and galacturonic acid *(Boluda-*

Aguilar & López-Gómez (2013). Experimental results demonstrated that 60 L ethanol was produced from 1000 kg of fresh lemon peels*(Boluda-Aguilar & López-Gómez (2013).*

ALTERNATE PATHWAYS FOR LIMONENE PRODUCTION AND ANALYSIS

Terpenes are considered as one of the most diverse classes of natural compounds and have a diverse range of applications in bio-pesticides, fragrances, pharmaceuticals, flavouring, and biofuels, etc.Limonene can be found in two optical forms in nature. (+) - limonene is the most widespread and well-known form of terpene and has various applications in industries like fragrance and flavour and in cosmetics.It can be found in more than 300+ plants' essential oils including lemon, orange and other citrus fruits *(Dictionary of natural products (2015).* By using limonene synthase, micro-organisms such as bacteria and yeast are being bioengineered to produce limonene.Wide variety of applications for limonene demands for stable large production of limonene volumes by metabolic engineering techniques and strategies for overexpressing of enzymatic pathway precursors.

CURRENT SCENARIO FOR PRODUCTION OF LIMONENE

Mostly (+)- limonene is present in the market due to its extensive use in various industries, currently being produced as a side product from the citrus juice industry. *Tranchida et al. (2013)* and *Sokovic et al. (2010)* stated that (+)- limonene content in citrus oil can be in the range of 70 – 98%. A study conducted by *(Lange 2015)* stated the production of limonene to be more than 60000 T/ year but it is always under suspicion due to harvest fails, which eventually fluctuates the price of orange peels as a substrate. Due to no disease management against bacterial diseases like Huanglongbing (HLB)*(Hodges and Spreen 2012).*, important production areas of Brazil and America is being affected resulting in a poor gross yield of citrus fruits.

Žiga Zebec et.al (2022) produce limonene by fermentation using bio-engineered E. coli cell factory. It's stated that (S) limonene and perillyl alcohol (derivative) is the most commonly found monoterpene produced by synthetic biology *(Alonso-Gutierrez J et,al(2013***).** In total nine genes were identified and encoded in a single synthetic gene cluster (SGC) which demonstrate required genetic intrinsic controls such as promoters, terminator,Ribosome Binding Sequence (RBS),and codon-optimized genes. All these components were assembled on plasmid pJBEI-6410, and enabled limonene production in 5ml culture. *(Alonso-GutierrezJ et,al(2013).* Same plasmid can be set upto 3.6 g/L of limonene productionat industrial scale*(Rolf J, Julsing MK et.al (2020).* These plasmids can enzymatically hydrolyse cellulose for production of glucose juice. The resultant glucose has to filtered, sterilised and used directly as substrate for terpenes production such as limonene (*Žiga Zebec et.al (2022)*). The enzyme blend Cellic CTec2 (Novozymes) was used which contained a blend of endoglucanase I (Cel7B), cellobiohydrolase I (Cel7A),and β-glucosidase for the degradation of cellulose 15. The enzymatic degradation was performed at 250 rpm, at 50 ˚C. The transformed cells were equipped with pJBEI-6410 in E. coli DH-10β in LB media containing

ampicillin*(Rolf J, Julsing MK et.al (2020).*The E. coli were analyzed in gas chromatography (GC) with the help of agilent technologies 7890 A GC system facilitated with 7693 autosamplers and FID detector. DB-Wax column (30 m; 0.32 mm; 0.25 μm) film thickness(30 m; 0.32 mm; 0.25 μm film thickness) split ratio was set at 10:1 (by 1 microlitre injection) and the temperature was set at 220 ˚C, pressure was adjusted at 9.2 psi and the helium gas flow rate was set at 1.5mL/ min. FID detector was maintained with a hydrogen flow rate at 30 ml/min. and at a temperature of 250 ˚C (*Žiga Zebec et.al (2022*)**).** This plasmid incorporated *E.coli* can be indulged in the production of limonene by various sources such as orange peels.

ANTI-MICROBIAL ACTIVITY OF ORGANIC ACIDS PRODUCED BY CITRUS WASTE

Organic acids are non-volatile in nature and can be extracted from citrus fruits for utilization as food decontaminants *(Mani-Lopez et al. 2012)*and contribute to maintaining the taste and quality of food *(Narayan et al. 2015; Scherer et al. 2012).*The various types of organic acids include malic, ascorbic, succinic, oxalic, and tartaric acid. The most frequently used organic acid is citric acid. These are extensively used in adjusting pH and acidulants favouring *(Smulders and Greer 1998; Ali et al.2015; Narayan et al. 2015).*Other than being a good source of OA's, citrus fruits are a good source of health-promising phytochemicals.*(Sdiri et al. 2012).*The phytochemical properties include antimicrobial and antioxidant properties.*(Mani-Lopez et al.2012; Rostamzad et al. 2011).* The phenolic group scavenges the free radicals present and prevents metabolic decay.*(Pandey and Rizvi 2009).*

Four bacterial strains [*Escherichia coli* (MTCC 43),*Bacillus subtilis* (MTCC 721), *Staphylococcus aureus* (MTCC 3160), and, *Klebsiella pneumonia* (MTCC 109) and two fungal strains i.e. *Aspergillus niger* (MTCC 404) and *Candida albicans* (MTCC 3017) were used to check the anti-microbial activity of the citrus juices.*(Shokri et.al (2011)).* When the results of OA concentration and the antimicrobial activity were superimposed the results shows the citric acid inhibitory activity against *S. aureus* (17.33 ± 0.70) at 2.5 mg and *K. pneumonia* (15.00 ± 0.00) at 2.5 mg*(Shokri et.al (2011)).Shokri et.al (2011)* reported the cause of the antimicrobial activity of citric acid is dueto change in pH of the microbial environment and factors like permeability of cell membrane being pH sensitive. *Blaszyk and Holley (1998)*reported inhibition in growth of common bacterial pathogens such as *E. coli, L. monocytogenes, Campylobacter spp, Arcobacter spp.* and *lactobacilli.* The citric acid was also proven to be a good fungal inhibitor with anti-fungal activity against *A. niger* (20 ± 0.00) and *C. albicans* (15 ± 0.00) at a concentration of 2.5 mg mL^{-1} whereas ascorbic acid demonstrated a high inhibitory zone for *K.pneumonia* (13.00 ± 0.00) at 2.5 mg*(Shokri 2011).*

Limonene demonstrates the wide variety of applications that differs in quality, quantity, price, and requirement *(Ciriminna et al.2014).*(+)- limonene has a wide application in the citrus flavouring industry such as soaps, soft drinks, and perfumes.Limonene demonstrates properties that can be utilized as an alternative for benzene, ethylbenzene, xylene and toluene as a solvent for gas and oil production*(Renninger et al.2008).*The presence of two double bond in the structure of limonene made it suitable for modification by adding the possibility of hydroxylation *(Wilbon et al. 2013).*Oxidized natural derivatives of limonene are used as flavouring agents. The addition of limonene can be helpful in lowering the viscosity of the diesel and lowering the cloud point *(Tracy et al. 2009).* Modification of limonene often results in the increased price of the product while limonene can be priced at 9–10 $/kg, but after transforming it into (-)- menthol its price elevates to 15–40 $/kg *(Lange 2015; Stuart Clark 1998).* Polymer of limonene is known as poly-limonene **(**Piccolyte C115) which can be achieved by citrus bio-oil *(Cimmino et al. 1999)*and can be used as adhesive resin. *(Barros et al. 2007)* has stated that terpene resin is suitable for drug delivery and other medical purposes. Limonene epoxide polymers can be used to produce varnishes, printing inks and metal coatings *(Firdaus et al.2011).* Furthermore, limonene can be converted to terephthalic acid, precursorused for polyethylene terephthalate (PET) plastic (Colonna et al. 2011). As a wide variety of industries get benefits from the limonene, it can be beneficial if produced on a large commercial scale.

CHAPTER – 3

MATERIALS AND METHODS:

Material used: Autoclaved distilled water, ampicillin(2mg/ml), autoclaved tips (1000µl, 200µl), pipettes (1000µl, 200µl), DMSO (10%), oil sample, Muller Hinton broth, Muller Hinton agar, autoclaved spreader, culture samples, test tubes, Whatman filter, forcep.

Chemicals used: Hexane, pure Ethanol, Petroleum ether, Dimethyl sulfoxide (DMSO(10%), Acetonitrile: water (1:1), Glacial Acetic acid

Raw material: Orange peels (sundried, slush/ grinded, acetic acid soaked)

Machines used: Centrifuge, Rotary evaporator, Lypholizer, Laminar air flow, -80˚C freezer, 4 ˚C Freezer, Soxhlet assembely (extractor, evaporator, heating mantle), Grinder, Hot air oven.

Microbial strains *Chromobacterium violaceum* (Gram-negative), *Salmonella enterica typhi* (Gram-negative), *Staphylococcus aureus* (Gram-positive), *Bacillus subtilis* (Gram- positive, *Escherichia coli ATCC 25922* (Gram-negative)

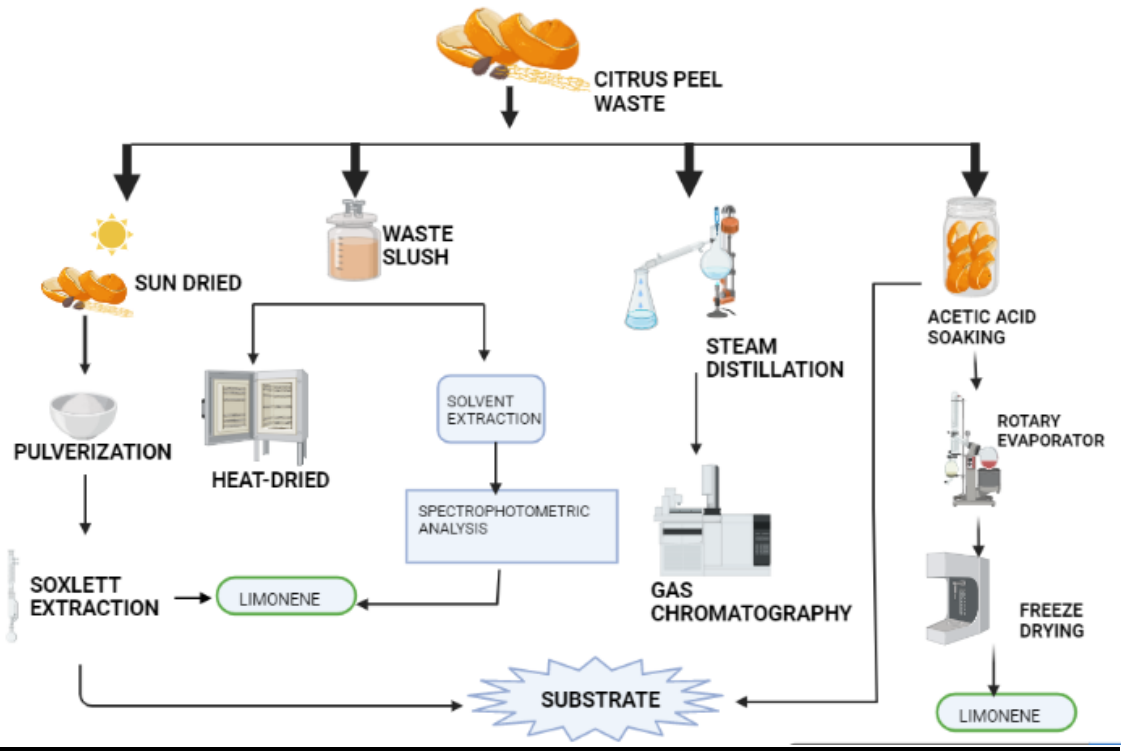


Figure 2*: The above figure shows the complete pathway of the methodological approach.*

The aim of the study is to find not only a pathway for complete utilization of orange peels but to discover a pathway that can be up-scaled into an industry that can be economically feasible for producing products that are commercially valuable. The products of focus are limonene and cellulase. Due to its antimicrobial nature, the presence of limonene in the substrate (citrus waste) is undesirable for the production of the enzyme via microbes.

Sample collection: Different samples were collected to quantify and qualify the amount of limonene produced. The samples were collected from two different locations:

(1) from the college mess (2) from the Solan local market (close to off-season).

SUNDRIED POWDER ANALYSIS

200g of the citrus waste (including orange peels, fibers, and seeds) were isolated from the fruit, and the waste was sundried for a week on aluminum foil. The resulting orange waste was collected and then pulverized using a grinder into a powder and stored in the refrigerator for the time being under 4 ˚C in falcon tubes. 25g of the powder was used for soxhlet extraction using hexane as a solvent. 500 ml round bottom flask was used for the soxhlet technique and the temperature was set at 70 ˚ C. Initially, the hexane imparted orangish-yellow color, but the solvent became colorless after 6-7 hours. For another 2 hours, the soxhlet was observed. After 9 hours the orangish-yellow solvent contained in the round bottom flask was removed.

USE OF PROCESSED ORANGE WASTE POWDER AS CARBON SOURCE FOR MEDIA

The colorless white orange waste powder was obtained from thimble weighing approx. 25 g. in small grain form which was pulverized into fine powder by grinding which can be used as a media component as a carbon source for microbial growth and eventually enzyme production.

The media was prepared with 3 different concentrations of soxhlet processed orange peel powder. Pre-treatment of powder was done by pulverizing the soxhlet obtained orange peel powder and then heating in the microwave at 200 ˚C to remove any kind of solvent remains from the powder.

Orange peel media was prepared with composition of components are as follow:

COMPONENT	AMOUNT (per 100ml)
PEPTONE	0.5g
SODIUM CHLORIDE	0.5g
YEAST EXTRACT	0.15g
ORANGE PROCESSED POWDER	(1) 0.15g (2) 0.30g (3) 0.50g
AGAR	1.5g + 2g = 3.5g

Table: Concentration of components used in preparing processed orange peel media

The media was autoclaved and poured in sterile petri-platesin laminar airflow. Four different bacterial cultures were selected to grow in these plates with different concentrations of processed orange powder to check the efficiency and potential of the processed orange powder as a carbon source for microbial growth. The microbes used for platting are as follows:

1) *Salmonella enterica typhi* (Gram-negative)
2) *Staphylococcus aureus* (Gram-positive)
3) *Bacillus subtilis* (Gram- positive)
4) *Escherichia coli* ATCC 25922 (Gram-negative)

The cultures were grown for 24 hrs to see the growth efficiency of the microbes on the orange processed media.

LIMONENE: ANTI-MICROBIAL TESTING

The oil extracted from the soxhlet extraction was tested for the anti-microbial activity by the agar well diffusion method.

Materials used:

Autoclaved water, ampicillin(2mg/ml), autoclaved tips (1000µl, 200µl), pipette, DMSO (10%), oil sample, Muller Hinton broth, Muller Hinton agar, autoclaved spreader, culture samples, test tubes, Whatman filter, forcep.

Methodology:

Muller Hinton broth (MHB) and Muller Hinton Agar (MHA) were prepared and a sample was taken with three different concentrations (1) 30mg (2) 50mg (3) 80mg (4) 100mg

(5)150mg(6)200 mg in 5 ml Eppendorf tubes, diluted and mixed with 1 ml DMSO (10%) solution. The Eppendorf tubes were stored in the refrigerator for the time being.

Culture preparation: two gram-positive and three gram-negative strains were selected for checking the anti-microbial activity of the oil namely

1)Chromobacterium violaceum (Gram-negative)

2)Salmonella enterica typhi (Gram-negative)

3)Staphylococcus aureus (Gram-positive)

4)Bacillus subtilis (Gram- positive)

5) *Escherichia coli* (Gram-negative)

The organisms were cultured in 10 ml of Muller Hinton Broth (MHB) overnight at 37˚C in the incubator shaker. The overnight grown cultureswere diluted with the broth by 10 times for re-culturing. i.e 100 µl of the culture was taken in the 1.5 ml eppendorf tube and diluted with 900 µl Muller Hinton broth (MHB) and mixed well for homogenizing the resulting solution. Then 100 µl of the resulting culture was transferred onto the Muller Hinton Agar (MHA) plates and spread equally by a sterilized spreader. After that, the wells were created by autoclaved 200 µl tips for consistency. In total, 5 wells were punctured for injecting

1) 20 µl of negative control of DMSO (10%)

2) 20 µl positive control (2mg/ ml)

3) 30 µl of 30 mg/ml sample oil solution

4) 30 µl of 50 mg/ml sample oil solution

5) 30 µl of 80 mg/ml sample oil solution

Then the plates were sealed with paraffin tape to protect them from external contamination and incubated at 37 ˚C overnight.

SLUSH ANALYSIS

Slush analysis was done in order to check the alternate path for obtaining /extracting the limonene. By providing an aqueous culture for the substrate. 250 g of the fruit waste including (peels, fibers, and seeds) were first pre-treated in a hot water bath at 70 ˚C for 30 minutes to loosen up the substrate for easy and efficient grinding. After grinding, thick

viscous slush was obtained and taken out in a beaker, and stored for analysis at 4˚C. Furthermore, three different falcon tubes were taken with 15 ml of slush sample each and mixed with hexane, ethanol, and petroleum ether separately in different falcons. The ratio of solvent and sample is 1:1. Sampleswere centrifuged at 8,000 rpm for 10 minutes at 4˚C. The yellowish supernatant phase was carefully separated by the debris and stored in different falcon tubes for spectrometric analysis of the samples by the double beam spectrophotometer method.

VINEGAR SOAKED METHOD

In search for a commercially cheap method for commercially valuable product, vinegar is the best suited option. The citrus waste was submerged in glacial acetic acid for 18 days in a cold and dark place. By using, rotatory evaporator limonene was isolated. The oil obtained was miscible in an acetonitrile-water 1:1 solution and was stored at -80˚C for lyophilization and conversion of the resulting material into powder form. The resulting powder was mixed in 10% DMSO for checking the anti-microbial activity of the powder.

CHAPTER - 4

RESULTS AND DISCUSSION:

SUNDRIED POWDER ANALYSIS

The resulting solvent from soxhlet was transferred into a beaker and placed in the hot water bath for 2 hours at 70˚C. The orangish-yellow-coloured oily substance was obtained weighing nearly 2.5 g. (Fig. 3 D)

The percentage oil yield from orange peel:

Percentage (%) limonene (oil) yield = (weight of oil /weight of citrus waste powder) x 100

= (2.5g / 25g) x 100

= 10%

(A) (B) (C) (D)

Figure 3: (A) soxhlet setup for extraction of limonene; (B)Limonene samples in hexane as solvent. (C) sundried orange powder as a substrate (D) limonene extract with processed orange powder as a by-product.

<u>DISCUSSION:</u> PERCENTAGE (%) LIMONENE (OIL) YIELD

In my experimental observations the percentage yield of limonene (oil) come up to be 10 %. When compared to the study by Lopresto et.al (2014) using Soxhlet extraction on 2 grams of dried lemon peels with hexane as a solvent at the solvent boiling temperature for 4 hours. Yields of D-limonene were observed to be 0.95% (100 $\cdot$ $_{g}$ limonene/$_{g}$ dried peels). Although the difference can be due to the difference in substrate composition and material handling.

UTILIZATION OF PROCESSED ORANGE POWDER AS THE CARBON SOURCE IN MEDIA FOR GROWTH OF MICROBES

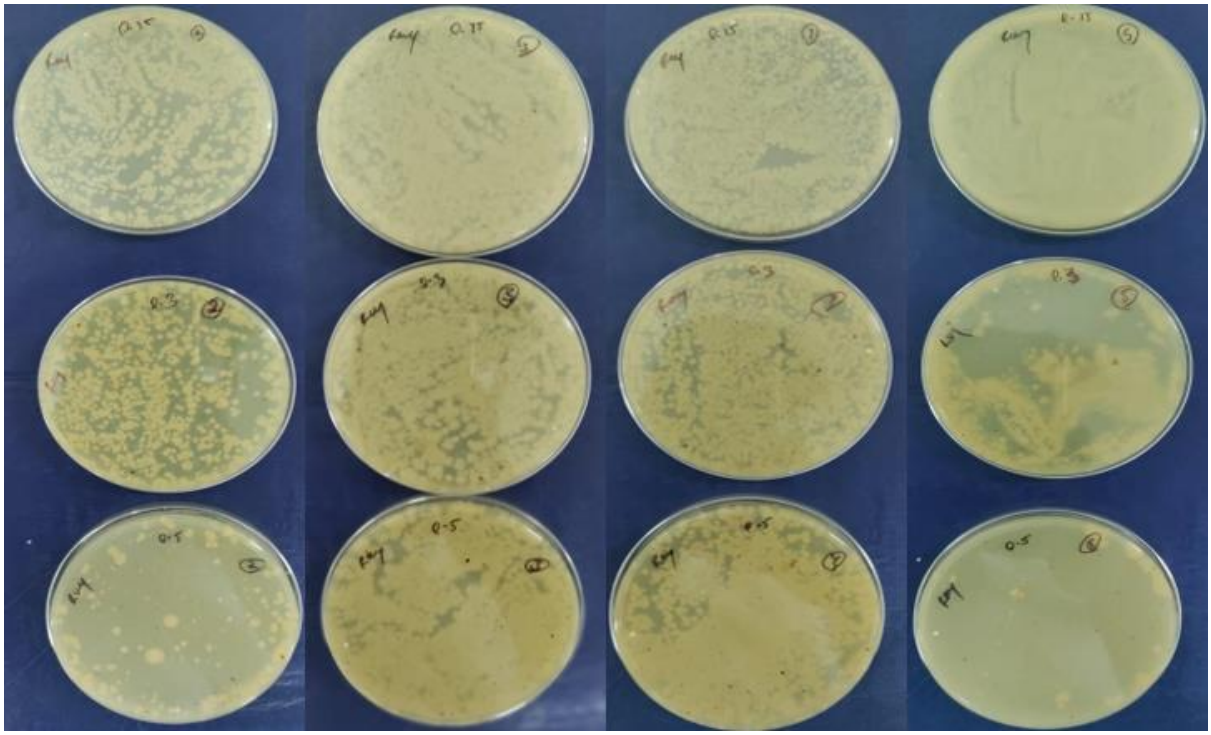
The processed orange powder was obtained as a by-product of the soxhlet extraction of limonene. Due to the anti-microbial activity of the limonene orange peels cannot be efficiently used as a potential carbon source for microbial growth. By extracting the limonene and other bio-oils present in the peels the orange waste became compatible to use as a carbon source for microbial growth. Orange peels were tested as an alternate carbon source for beef/ meat extract. With the concentration comparable with the amount of beef/ meat extract use in 100 ml media (0.15g /100ml) and other alternate concentrations to check the optimized concentration for orange peels (0.3g /100ml, 0.5g/ 100ml).The microbes used were:

1*)Salmonella enterica typhi* (Gram-negative) (plate A)

2*)Staphylococcus aureus* (Gram-positive) (plate B)

3*)Bacillus subtilis* (Gram- positive) (plate C)

4*)Escherichia coli* (Gram-negative) (plate D



(A) (B) (C) (D)

Figure 4: Different culture growth (A:Salmonella enterica typhi; B: Staphylococcus aureus; C: *Bacillus subtilis*; D: *Escherichia coli) on different concentration (*Top: *0.15g/100ml;* Middle: *0.30 g/100ml;* Bottom: *0.50 g/100ml) of processed orange peels as carbon source*

Salmonella enterica typhi (plate A), growth was comparable at concentrations (0.15g/100ml, and 0.3g/100ml) but showed inhibition at concentration (0.5g/100ml). *Staphylococcus aureus* (plate C) demonstrated equal growth at different concentration (0.15g/100ml, 0.3g/100ml, 0.5g/100ml) after 24 hours of growth. *Bacillus subtilis* (plate C) showed equal growth at different concentration (0.15g/100ml, 0.3g/100ml, 0.5g/100ml) after 24 h growth. In case of *Escherichia coli* (plate D) the growth gradually decreased with increasing concentration of processed orange powder concentration. The growth of *E.coli* ATCC 25922 at different concentrations were (0.15g/100ml > 0.3g/100ml > 0.5g/100ml).

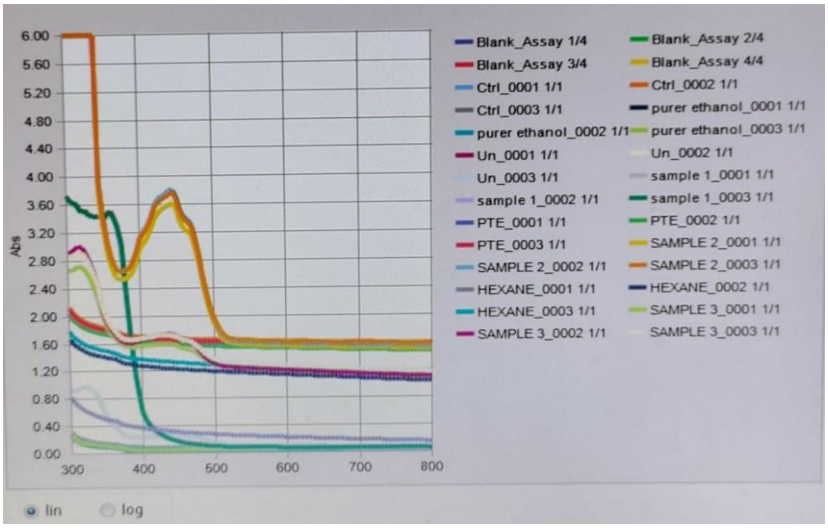
DISCUSSION: Orange peel waste is rich in cellulose, hemicellulose, pectin, carbohydrates, protein, and lipids because of its rich composition it can be used to grow microbes for

enzymatic production *(Rivas et al. 2008).* When replaced beef extract with orange peels as a carbon source, the microbes grow equally efficiently as before. In some cases, the inhibitory carbon source concentration was observed as we increase the processed orange peels powder concentration from 0.15g/ 100ml > 0.3 mg/100ml > 0.5g/100ml (fig.4(D)). Optimisation is require to increase the efficiency of the carbon source utilization

SPECTROPHOTOMETRIC ANALYSIS

UV-Vis spectral analysis of the samples was done. Due to cyclohex-1-ene group C=C bonds π->π* transition at absorbance is shown at 200 nm to 360 nm and prop-1-en-2yl group C=C bonds π ->π* transition another absorbance is shown at 380 nm to 500 nm, which confirms the orange color of the solution. *(Ketaki and Chittaranjan (2020))*

A range was set between 300nm to 800 nm to check the compound activity at different nano-meter ranges using 96- well plate method and compared the results with the provided information from the literature. Peaks were observed between the range of 380 to 500 nm confirming the presence of limonene in the sample. In addition, an orange-like fragrance was observed which can be used as an olfactory indicator of limonene's presence in different sample compounds.Pure ethanol, hexane, and petroleum ether were used as mixing solvents for the compound. After that, 450 nm was set to check the presence of limonene in the compound. Peaks shown in the sample indicate the presence of limonene. (Fig. 5)



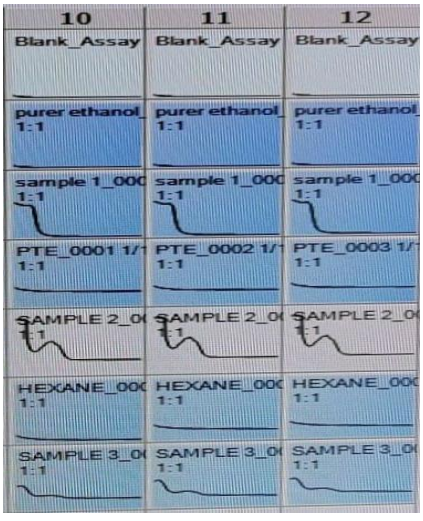


Figure 5: the spectral range of absorbance was checked by setting range from 300nm to 800nm for samples having ((a) ethanol (b) petroleum ether (c) hexane) as separate solvents.

For further, confirmation double beam spectrophotometric analysis was done at 280nm and 450nm for ethanol sample, and 230nm, 280nm, 300nm for petroleum ether sample to check the deviation and presence of the compound. Distilled water was taken as blank to calibrate the data. (Table 2)

	At 450 nm	**At 280nm**
Ethanol	0.000	0.051
Ethanol sample	0.287	3.960
Deviation (▲)	0.287	3.909

Table 2: Using double beam spectrophotometer, limonene containing samples in ethanol as solvent were tested for the deviation in absorbance from pure ethanol control

	At 230 nm	**At 280 nm**	**At 300nm**
Petroleum ether	3.689	2.251	0.084
Petroleum ether sample	4.366	4.189	4.181
Deviation (▲)	0.677	1.938	4.097

Table 3: Using double beam spectrophotometer, limonene containing samples were tested for the deviation in absorbance from pure petroleum ether as control

DISCUSSION

A study by *(Ketaki and Chittaranjan (2020))* stated that using UV-Vis spectral analysis of the samples, the absorbance is shown at 200 nm to 360nm due to cyclohex-1-ene group C=C bonds π->π* transition. and another absorbance is shown at 380 nm to 500 nm due to prop-1-en-2yl group C=C bonds π ->π* transition which confirms the orange color of the solution. *(Ketaki and Chittaranjan (2020)).* In my study the Peaks were observed between the range of 380 to 500 nm confirming the presence of limonene in the sample. In addition, an orange-like fragrance was observed which can be used as an olfactory indicator of limonene's presence in different sample compounds.

Using ethanol and petroleum ether samples at 280 nm using a double beam spectrophotometer, a deviation value of absorbance was shown by the samples compared when compared to their pure solvent form. The deviation value in case of ethanol samples were 3.909 (table 2) and for petroleum ether samples the deviation was observed to be 1.938.

(table 3) These results demonstrated prop-1-en-2yl group C=C bonds π ->π* transition due to absorption which confirms the orange color of the solution

A higher deviation in absorbance was seen in petroleum ether samples at 300 nm with a value of 4.097 when compared to the pure petroleum ether as a solvent which confirms cyclohex-1-ene group C=C bonds π->π* transition due to absorption.

LIMONENE: ANTI-MICROBIAL TESTING

The antimicrobial testing was done using two gram-positive and three gram-negative strains were selected for checking the anti-microbial activity of the limonene present in oil namely

1)Chromobacterium violaceum (Gram-negative)

2)Salmonella enterica typhi (Gram-negative)

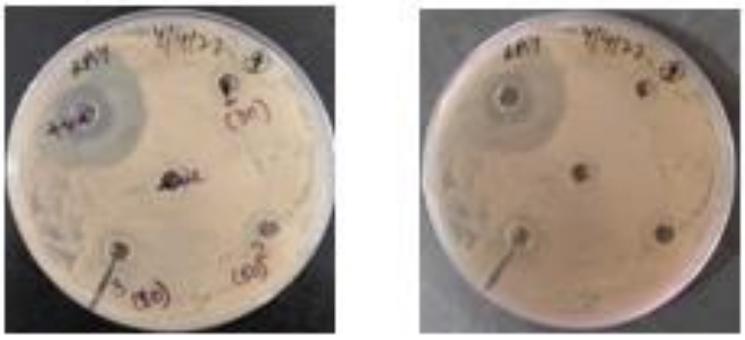
3)Staphylococcus aureus (Gram-positive)

4)Bacillus subtilis (Gram- positive)

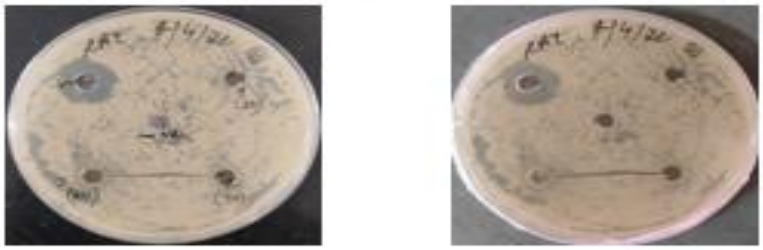
5)Escherichia coli (Gram-negative)

Ampicillin (2mg/ml) was used as a positive control for anti-microbial checking. And DMSO (dimethyl sulphoxide) was used as the negative control. At low concentrations of 30mg/ml, there was no zone of inhibition in any of the plates. At concentration of 50 mg/ml, no prominent zone of inhibition was seen although in *Bacillus subtilis* (gram- positive) (plate no.4) scattered colonies were observed. At concentration of 80 mg/ml a small zone of inhibition of radius 2 mm was observed on *Staphylococcus aureus* (gram-positive) (plate no.3).

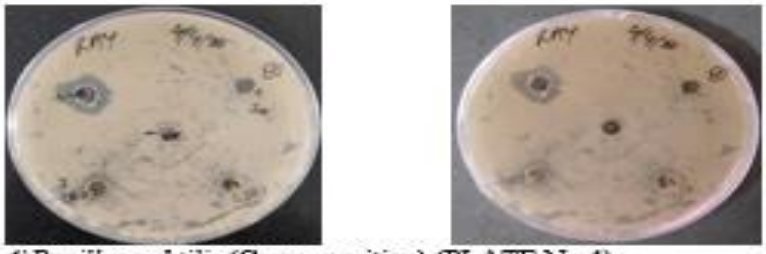
1)Chromobacterium violaceum (Gram-negative) (PLATE No. 1)



2) Salmonella enterica Typhi (Gram-negative) (PLATE No. 2)



3)Staphylococcus aureus (Gram-positive) (PLATE No.3)



4)Bacillus subtilis (Gram- positive) (PLATE No.4)

Figure: Above images shows the anti-microbial activity of limonene at (i) 30mg/ml (ii) 50mg/ml (iii) 80mg/ml (iv) Negative control - DMSO (10%) (v) positive control – Ampicillin (2mg/ml)

As the results were not prominent, the experiment was performed again at a higher concentration of limonene samples (100mg/ml, 150mg/ml,200mg/ml) to check the anti-microbial activity of the limonene. At 100 mg/ml no zone of inhibition is formed on any of the plates except*Salmonella enterica Typhi* (gram-negative) (plate no. 2) anda scattered colony near the 100mg/ml well was formed but no prominent zone of inhibition was present. In case of 150 mg/ml, no zone of inhibition was observed except, scattered colonies were formed on the *Salmonella enterica Typhi* (gram-negative) (plate no. 2) and *Bacillus subtilis* (Gram-positive) (PLATE No.4). Ina concentration of 200 mg/ml scattered colonies were formed at *Salmonella enterica Typhi* (Gram-negative) (plate no. 2) but no prominent zone of inhibition was formed. In addition, negligible or less dense colonies were formed at

Staphylococcus aureus (Gram-positive) (PLATE No.3) and *Bacillus subtilis* (Gram- positive) (PLATE No.4)

1)Salmonella enterica Typhi (Gram-negative)

2)Staphylococcus aureus(Gram-positive)

3)Bacillus subtilis (Gram- positive)

4)Escherichia coli (Gram-negative)

Figure: The above images show anti-microbial activity of limonene at concentrations: (i)100 mg/ml (ii)150 mg/ml (iii)200mg/ml (iv) Negative control - DMSO (10%) (v) positive control – Ampicillin (2mg/ml)

DISCUSSION

(Shokri et.al (2011)) used four bacterial strains [*Escherichia coli* (MTCC 43), *Bacillus subtilis* (MTCC 721), *Staphylococcus aureus* (MTCC 3160), and, *Klebsiella pneumonia* (MTCC 109) and two fungal strains i.e. *Aspergillus niger* (MTCC 404) and *Candida albicans* (MTCC 3017) were used to check the anti-microbial activity of the citrus waste *(Shokri et.al (2011))*.

Although limonene was extracted by the soxhlet method the efficiency was not convincing enough even at higher concentrations. There was no prominent zone of inhibition. The volatile nature of limonene and the high density of oil can be the reason behind the unsatisfactory result. in addition, during the evaporation of hexane, the oil incorporated method can be utilized to overcome the volatile nature of limonene. Steam distillation can also be utilized to get a better result than the soxhlet method due to volatile nature of limonene.

VINEGAR SOAKED METHOD

After 18 days the glacial acetic acid (orangish-yellow color) was obtained and isolated in a beaker for analysis. The change in color was a clear indication of isolation of pigments and compounds. the isolated solution was freezed at -80˚ C for 24 hours and then lypholized to get light yellowish powder. The powder was scraped out from the round bottom flask and then the anti-microbial activity was checked.

DISCUSSION

Although the method showed antimicrobial properties and can be use in cleaning, the technique cannot be ideal to upscale due to increase in cost due to lyophilisation. But on contrary, this technique showed potential in achieving good grade antimicrobial activity and surface cleaning property.

CHAPTER - 5

CONCLUSION

The orange peels are rich in components like cellulose, hemicellulose, fats, and pectin. which make them a suitable candidate for manufacturing different cmeercially valuable enzymes and compounds. the antimicrobial nature of limonene which is present in bio-oil of orange peels makes it hard to decompose or degrade in nature, when use as landfills creating problems like releasing green-house gases like (CO_2 and CH_4) resulting in global warming and burning which results in the release of dioxins. An efficient and commercially beneficial method includes first extraction and isolation of limonene as it has applications in various industries (like solvent , fragrance, perfume, polymer, anti-microbial, flavouring, preservatives, cleaning, industryetc.) and then utilizing the processed orange peel waste to manufacture different enzymes like pectinase, lipase, protease, cellulase, amylase etc. the processed orange peel powder can also be used as a carbon source for microbial growth.

Soxhlet method can be used to extract limonene but is not efficient in securing the anti-microbial activity of the compound, due to the volatile nature of the compound, incorporation of different techniques may result in betterment in securing the activity of the limonene even after extraction. Steam distillation can be use instead of soxhlet for extraction of volatile compound like limonene and can result in better yield of the product and its concentration.

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