A Review on Bioethanol Production from the Orange Peel Waste (OPW) Using Cellulolytic Soil Bacteria

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Abstract –To avoid economic impact and environmental effect of agricultural waste, the reuse of the agricultural waste is needed. For this purpose, the naturally available cheap soil microorganisms with potential application can be used. Many soil microbes have cellulase enzyme which can act on lignocellulosic waste material. The current research on producing bioethanol are aimed to reduce the production cost using low-cost substrates and enzymes. In line with this, in this study the orange peel waste will be used to produce bioethanol using the soil isolated microorganisms in the cost-effective way. The pretreatment methods with various approaches to make the substrates more exposed and further fermentation process would be very much cost effective. Utilizing the natural microorganisms with cellulase activity in producing the biofuel from OPW is a value-added biotechnological method in future.

Keywords- Bioethanol ,cellulolytic soil microbe, fermentation , lignocellulosic waste, orange peel waste, pretreatment.

1.Introduction –

Bioethanol is a plant derivative and a "renewable" energy source. The production and combustion of ethanol completes a full cycle releasing carbon dioxide and water [1]. Ethanol is one of the important industrial chemicals which could be used to synthesise other organic compound or used as solvent, as additive in automotive gasoline. It is also an intoxicating component used in alcoholic beverages such as distilled spirits, wine, beer etc. Ethanol normally used as primary fuel in the form of an additive in petrol powered engines to run automobiles. Ethanol is the most prominent biofuel in the world, but it is also a critical ingredient for pharmaceuticals, bio-plastics, bio-chemicals, food and many other necessities.

Bioethanol is the most common and worldwide used biofuel in the transportation sector, which could directly be utilized as a fuel either in a pure form or blended with gasoline. Bioethanol is high-octane fuel and it lessens the release of carbon monoxide and smog [2].Hence the biofuel reduces the greenhouse emission by replacing fossil fuels.

Biomass of Agricultural feedstocks from crops such as hemp, corn, potato etc which normally produce ethanol as the By-product. United States of America (62.2%) and brazil (25%) are the world's top 2 producers of ethanol. In 1978 Fiat 147 was the very first production car to run on pure ethanol fuel.

Producing ethanol fuel is not a new thing in India as the ministry of petroleum and natural gas (MoPNG)first proposed ethanol as automotive fuel in 2003.Due to India's carbon foot print and future environmental impact in 2008 ministry of new & renewable energy penned down the national policy leading to a target of 20% blending of bioethanol which has helped India's dependence on gulf nations for crude oil (India used to spend around 7 lakh crores in importing crude oil).

To some extent we can also use ethanol in diesel. Around 15% blending of ethanol-diesel is known as e-diesel. Sweden currently uses e-diesel to run trucks. But, using ethanol in a diesel engine is still not an efficient alternative in comparison to petrol due to its low flash point and safety issues.

According to latest reports of 2019 there are many Challenges of producing bioethanol in India such as - Ethanol fuel is produced using biomass from agricultural produce therefore having a high dependency on agriculture.

They also face challenges due to its impact on agriculture and land use. As farmers will have to clear more lands which will affect the native wildlife. Ethanol also finds usage in pharma, beverage and chemical industries hence facing high competition from these sectors [3].

1.1 Orange peel waste (OPW) – Cellulose is the most abundant lignocellulosic biomass on the earth [4].Plant biomass contains cellulose as the major component. Which accounted for about 50% of the dry weight of plant biomass and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes [5]. It has been reported that huge amount of agricultural and industrial cellulosic wastes has been accumulating in environment. Cellulose has attracted worldwide attention as a renewable resource that can be converted into

bioenergy and bio- based products [6]. Celluloses are observed as the most important renewable resource for bioconversion. It has been become the economic interest to develop an effective method to hydrolyse the cellulosic biomass [7].

Citrus producing industry in India is the third largest fruit industry of the country after mango and banana. India ranks ninth among top orange producing countries contributing 3% to the world's total orange production. In India, citrus is grown in 0.62 million ha. area with the total production of 4.79 million tonnes. Oranges are mostly grown in the states of Maharashtra, Madhya Pradesh, Arunachal Pradesh, Tamil Nadu, Assam, Orissa, West Bengal, Rajasthan, Nagaland, Mizoram. Important mandarin orange varieties cultivated in India are Nagpur Santra, Coorg Santra, Khasi Santra, Mudkhed, Shringar, Butwal, Dancy, Kara (Abohar), SZ-IN-COM, Darjeeling Mandarin, Sumithra mandarin, Seedless-182 and Kinnow mandarin [8]. Orange peels being is the most suitable lignocellulosic waste which are used to producing bioethanol with the use of enzymes and fermentation technology [9]. Depending on the environmental conditions it was determined that the orange peel take up to six months to get completely decompose, although in drier environments like Central Oregon, oranges can last indefinitely. The thing is, even though these things decompose more quickly than materials like plastic or glass, they still stick around for a while [10]. The conversion of lignocellulosic biomass to bioethanol and other value-added products is promising because of its abundance and cost-effectiveness. Moreover, it does not affect the land use and food production.

The term Lignocellulosic biomass is nothing but lignin, cellulose and hemicellulose that constitute the plant cell wall [11].Currently, lignocellulose is being produced from wood residues, agricultural residues, food industry residue, grasses, domestic wastes, municipal solid wastes, and non-food seeds [12, 13, 14].

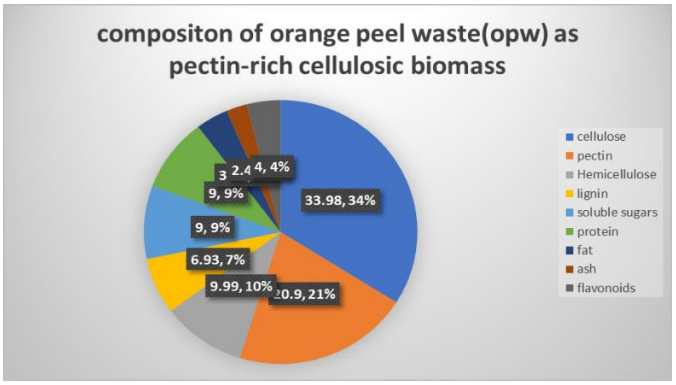
The lignocellulose wastes (LCW) are largest renewable bioresource reservoir on earth that is being wasted as pre- and post-harvest agricultural wastes [15]. Thus, many steps need to be adopted for use of these renewable resources for the production of bioenergy products. The US government has planned the production of 21 billion gallon of biofuels by 2022 [16].

Biofuel production from lignocellulosic biomass reduces the emission of greenhouse gases as reported currently in 2019 [11].

1.2 Composition of orange peel waste (OPW)cell wall: -

The OPW cell wall is thick due to association of lignin, cellulose and hemicellulose and other components. In order to break down this hindrance, pre-treatment is an essential step which helps in the providing access for the enzymes to act upon the lignocellulosic biomass, hence increases the digestibility and product yield [17].

Orange peel waste is a valuable lignocellulosic biomass or feedstock for the production of bioethanol due to the richness in carbohydrates (fermentable sugars) and low lignin content.



Before subjection to enzymatic hydrolysis, pre-treatment of biomass can increase the rate of hydrolysis by 3–10fold. Pre-treatment brings physical, biological, and chemical changes to biomass structure; therefore, it is very important to consider the type of pre-treatment. pre-treatment is the second most costly process at industrial level.

Lignocellulosic biomass (orange peel) is composed of carbohydrates such as cellulose, hemicellulose and aromatic polymer lignin, pectin (galacturonic acid) and crude fibre, fat, low molecular weight compounds such as limonene. The composition of these carbohydrates varies in various lignocellulosic biomasses. The feedstock should be more in carbohydrates and less in lignin for sustainable bioethanol production.

1.3 Microbes-soil ecosystem is rich in numerous and diverse microorganisms which can be classified as bacteria, actinomycetes, fungi, algae, protozoa and viruses. Each of these groups has different characteristics that define the organisms and different functions in the soil it lives in [18]. Bacteria with cellulase activity has a capability to degrade cellulose to simple sugars components.

Cellulose is commonly degraded by an enzyme called cellulase. Cellulase are proteinaceous molecules called enzymes. These enzymes are produced by several microorganisms, commonly by bacteria and fungi [19]. Cellulase is an important and essential kind of enzyme for carrying out the depolymerization of cellulose into fermentable sugar [6]. Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on Cellulosic matters [20] . Cellulose

degrading microorganisms can convert cellulose into soluble sugars either by acid and enzymatic hydrolysis. Thus, microbial cellulose utilization is responsible for one of the largest materials flows in the biosphere [21].Increasing knowledge of mode of action of Cellulase, they are used in enzymatic hydrolysis of cellulosic substances [22].

Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulosic sources, cellulose containing raw materials and waste products that are not exploited or which could be used more efficiently [23]. Cellulases are used in the textile industry for cotton softening, denim finishing, in laundry detergents for colour care, cleaning, in the food industry for mashing, in the pulp and paper industries for drainage improvement and fibre modification, and they are even used for pharmaceutical applications [24]. Over all the cellulose enzymes will be commonly used in many industrial applications and the demands for more stable, highly active and specific enzymes will also grow rapidly [25].Cellulases form bacteria are also more effective catalysts. They may also be less inhibited by the presence of material that has already been hydrolysed. The greatest potential importance is the ease with which bacteria can be genetically engineered [26].

Bacteria has high growth rate as compared to fungi has good potential to be used in cellulose production [23]. Some bacterial species such as Cellulomonas species, Pseudomonas species, Bacillus species and Micrococcus have cellulolytic property [27]. A large number of microorganisms are capable of degrading cellulose, only a few of them produces significant quantities of cell-free bioactive compounds capable of completely hydrolysing crystalline cellulose in vitro.

Many researchers have reported that the degradation of cellulosic materials, but few studies have examined which microorganisms had met the industrial requirement [20].Among bacteria, Bacillus species produce a number of extracellular enzymes including amylases, proteinases, and polysaccharide hydrolases [28]. For understanding the mechanism of cellulose degradation by cellulase, it is necessary to isolate, purify and characterize this enzyme.

Types of cellulases-3types of cellulases involved in hydrolysis of cellulose are

Endo-beta -glucanase, Exo-beta-glucanase, Beta-glucosidase.

- Endo-beta-1,4-glucanases or endoglucanases act by cutting at random at internal amorphous sites in the cellulose polysaccharide chain generating oligosaccharide of various lengths. These enzymes are glycoproteins with mol wt from 5,300-14,500. They are referred to as carboxymethylcellulases.
- Exo-beta-1,4-glucanase or exoglucanase acts on reducing and non-reducing ends of cellulose polysaccharide chain liberating either glucose(glucanohydrolases) or cellobiose(cellobiohydrolase) as major products.
- Beta-glucosidase or cellobiases hydrolyse soluble cellodextrins and cellobiose to glucose [29]

But the problem is the use of commercial enzymes which helps in converting the cellulose to simple sugars is a cost effective. As enzymes cellulase are too costly to be used in large amount. Hence isolating the soil microbes with cellulase activity and using them is a best method.

Currently the USDA scientists are working on new ways to breakdown the plant fibres or conversion into biofuel, hence they are using cow's rumen enzyme called feruloyl esterases (FAEs) for genetic manipulation [30].

2. Process of bioethanol production –

Bioethanol production from any lignocellulosic biomass involves three major steps such as, pre-treatment, hydrolysis, and fermentation.

1) **Pre-treatment** -It is a process where the recalcitrant cell wall is disrupted to make the carbohydrates accessible for hydrolysis. Pre-treatment method basically disrupts the hydrogen bonds, cross link matrix disruption, increases the porosity as well as surface area of cellulose. These are the three tasks performed by suitable pre-treatment method. The result of pre-treatment also differs based on the difference in the cell wall components ratio [31, 32].Pre-treatment method is an efficient, effective, economical process which includes the use of cheap chemicals in very low quantity with minimal energy requirement and consumption which also avoids denaturation of cellulose and hemicellulose. There are many methods of pre-treatment which could be broadly divided into four types such as physical, chemical, physicochemical, biological [33, 34, 35].

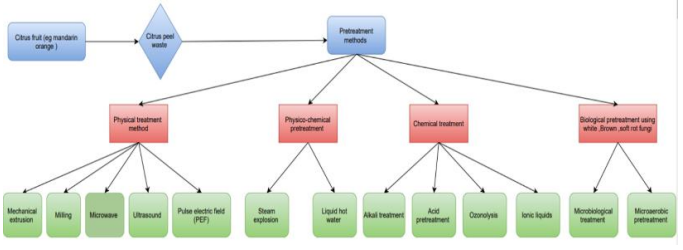


Fig:-Pretreatment methods of lignocellulosic biomss [36]

a) **Physical method**-which includes Mechanical extrusion, Milling, Microwave, Ultrasound pre-treatment, PEF etc.
   i. **Mechanical extrusion** -this process also known as sugar recovery from biomass. It has many

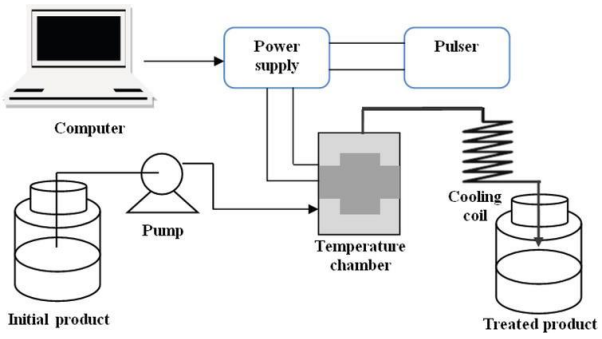
advantages such as no degradation of products, controllable environment, and high throughput. It's a conventional process where the biomass will be heated above 300 ^{0} C under shearing mixing. The high-energy requirement and shearing forces make this process costly and difficult to scale up at industry level [37] .Two types of extruders such as Single screw extruder and twin-screw extruder are used. Single screw extruder which has three screw elements such as forward, kneading, and reverse, with minimum shearing and mixing, bulk material of varying pitches and lengths can be transported by forward screw element. Twin screw extruder which does multiple tasks at the same time like, shearing, grinding, mixing reaction, drying, and separation. Short-time extruders provide fast heat transfer, proper mixing, and increased shear. Material which passes through the extruder barrel disturb the structure of biomass and thus exposing more surface for enzymatic hydrolysis [38, 39, 40].

ii. **Milling** -Milling can be performed to render lignocelluloses more amenable to cellulases. There are many types of milling based on different materials used, For wet material, colloid mill, dissolver, and fibrillator are suitable, whereas for dry materials hammer mill, extruder, cryogenic mill, and roller mill are used. For both wet and dry material, ball milling can be used. For waste paper, hammer milling is the most suitable pre-treatment option [41, 42, 43]. Chipping involves the breakdown of biomass into 10–30 mm pieces, whereas milling and grinding decrease the size of biomass to 0.2 mm. Chipping prevents heat and mass transfer loss, while shear forces generated during milling reduces the particle size and cellulose crystallinity. The biomass type and time span of milling are the determining factors for decreasing the crystallinity of cellulose, enlarging specific area [37].

iii. **Microwave** -The best method used for plant biomass pre-treatment is microwave irradiation. This pre-treatment method has several advantages such as ease of pre-treatment, minimal generation of inhibitors, and less energy requirement, increased heating capacity, short processing time. Researchers from Kyoto University, Japan in 1984 had reported microwave irradiation in closed container for first time [44].To avoid large temperature gradients, microwave is a good choice as it uniformly distributes heat which also avoids degradation of lignocellulosic material into humic acid and furfural. As microwave irradiation is performed at high temperature, therefore, closed containers are required to achieve high temperature. Three properties, namely, penetration, reflection, and absorbance are exhibited by microwave. Microwave passes through glass and plastic, absorbed by water and biomass, whereas microwaves are reflected by metals. Based on these properties, microwave reactors can be divided into two types, one that allows the passage of microwaves, whereas the other kind reflects the microwaves. Glass or plastic is the building material of the first type of microwave reactors, whereas the second types of reactors are composed of steel. Through quartz windows, microwaves can enter into the reactor as these are placed in the reactor. Closed, sealable, pressure-resistant glass tube container having gasket made up of Teflon can be used for the high temperature, i.e., 200°C, for microwave irradiation pre-treatment. Sensors are used to control and ensure temperature inside the microwave. Teflon-coated sensors are a good choice because of the thermostability, corrosion-free nature, and zero absorbance properties. In a microwave oven, Teflon vessels are used by some scientists due to its advantageous properties [45] [46]. Irradiation with microwave at low energy is an easy option giving high heat in short duration and it degrades lignocellulose structure with minimum generation of inhibitors [37].

iv. **Ultrasound pre-treatment** - ultrasound waves affects lignocellulosic biomass both physically and chemically. It forms small cavitation bubbles causing rupturing of cellulose and hemicellulose and thus increases accessibility to cellulolytic enzymes for conversion into simpler reducing sugars. Ultrasound frequencies of 10–100 kHz have been used by various researchers to treat lignocellulosic biomass [47].

v. **Pulse electric field (PEF)-** In this method, Sample was placed between two parallel plate electrodes, and the strength of electric field is given as E = V/d, where V and d are voltage and distance, respectively, between plate electrodes. Hence the pores are created in the cell membrane due to which cellulose exposes to such agents that cause its breakdown by entering into the cell. High voltage ranging between 5.0-20.0 kV/cm is applied in a sudden burst to biomass for nano to milliseconds.



Pulse electric field_[11]

Setup of pulse electric field consisted of pulse generator, control system, data acquisition system, and material handling equipment_[17]_.Advantage of this treatment is the simple design of the instrument. Short duration of pulse time saves the effort and energy [48].Pulse electric field can increase hydrolysis rate by exposing cellulose to catalytic agents [17, 49].

b) Chemical pre-treatment –

i. **Dilute Acid pre-treatment**-In this pre-treatment, acids are used to pre-treat lignocellulosic biomass. The generation of inhibitory products in the acid pre-treatment renders it less attractive for pre-treatment option. Hence this method is not used always. Furfurals, aldehydes, 5-hydroxymethylfurfural, and phenolic acids are the inhibitory compounds that are generated in huge amount in acid pre-treatment. There are two types of acid treatments based on the type of end application. One treatment type is of short duration, i.e., 1–5 min, but high temperature > 180°C is used, and the second treatment type is of long duration, i.e., 30–90 min, and low temperature < 120°C is utilized. Due to hydrolysis by acid treatment, separate step of hydrolysis of biomass can be skipped, but to remove acid, washing is required before the fermentation of sugars [48, 50].To treat lignocellulosic biomass, concentrated acids are also used. Most commonly used acids are sulfuric acid or hydrochloric acid. In order to improve the process of hydrolysis for releasing fermentable sugars from lignocellulosic biomass, acid pre-treatment can be given. For poplar, switch grass, spruce, and corn stover, sulfuric acid pre-treatment is commonly used. Reducing sugars of 19.71 and 22.93% were produced as a result of the acid pre-treatment of Bermuda grass and rye respectively. At 4 wt. % concentration of sulfuric acid, pre-treatment is preferred because of less cost and more effectiveness of the process. Dilute sulfuric acid causes biomass hydrolysis and then further breakdown of xylose into furfural is achieved. High temperature favours hydrolysis by dilute sulfuric acid [51].Removal of hemicellulose is important to increase glucose yield from cellulose, and dilute sulfuric acid is very effective to achieve this purpose.It is necessary for an economical biomass conversion to achieve high xylan-to-xylose ratio. One-third of the total carbohydrate is xylan in most lignocellulosic materials. There are two types of dilute acid pre-treatments, one is characterized by high temperature, continuous flow process for low solid loadings, and the other one is with low temperature, batch process and high solid loadings [52]. Temperature and solid loadings for the first type are >160°C and 5–10% respectively, and for the second type the temperature and solid loadings are around<160°C and 10–40% respectively [53].Besides sulfuric acid and hydrochloric acid, other acids like oxalic acid and maleic acid are also used for the pre-treatment of lignocellulosic biomass. Other advantages include less toxicity to yeast, no odour, more range of pH and temperature for hydrolysis, and no alterations in glycolysis [54].

ii. **Alkali pre-treatment**-Apart from acids there are few bases used for pre-treatment of biomass. Lignin contents greatly affected by alkaline treatment. As compared to other pre-treatment methods, alkali treatment requires less pressure and temperature and ambient condition, but it's a time-consuming process as it can take hours to days. Degradation of sugar in alkali treatment is less than that by acid treatment, and also the removal and recovery of caustic salt are possible and easy in case of alkali treatment. Ammonium, sodium, calcium, and potassium hydroxides are used for alkaline pre-treatment, but among this sodium hydroxide is the most commonly used alkaline pre-treatment agent, whereas calcium hydroxide is the cheapest yet effective among all other alkali agents for pre-treatment. By neutralizing calcium with carbon dioxide, calcium can be recovered easily in form of insoluble calcium carbonate. Using lime kiln technology, calcium hydroxide can be regenerated [55]. Apparatus required for alkali pre-treatment is basically temperature controller, a tank, CO2 scrubber, water jacket, manifold for water and air, pump, tray, frame, temperature sensor, and heating element. The first step of pre-treatment consists of making lime slurry with water. The next step is spraying of this slurried lime on biomass; after spray, store the biomass for hours or, in some case, days. Contact time can be reduced by increasing temperature [56, 57, 58].

iii. **Ozonolysis pre-treatment**-degrades lignin content in the biomass [59] .It is an environment friendly process as it does not release toxic compounds and thus does not affect the hydrolysis and fermentation process [60].

iv. **Ionic liquids pre-treatment**-This methods are frequently used in recent decades. solvents are made up of ions that have low melting points (below 100 °C), low vapor pressure, high thermal stabilities and high polarities [61].

c) Physico-chemical pre-treatment-

i. **Steam explosion**-In this method, high-pressure saturated steam is used to treat lignocellulosic biomass, and then suddenly pressure is reduced, due to which lignocellulosic biomass undergoes explosive decompression.

This is also known as auto-hydrolysis. Steam explosion process involves combination of mechanical forces (pressure drop) and chemical effects (autohydrolysis of acetyl groups of hemicellulose). In this process, biomass is provided with saturated steam at high pressure (0.7–4.8 MPa) and high temperature (160–260 °C) with 0.69–4.83 MPa pressure is provided for few seconds to minutes, this triggers hydrolysis of hemicellulose and at the end explosive decompression, terminated the whole process

[62]. Cellulose hydrolysis potential increases due to the cellulose degradation and lignin transformation caused by high temperature. During the steam explosion pre-treatment, acid and other acids formed, which played their role in the hydrolysis of hemicellulose [63]. Fragmentation of lignocellulosic material occurs due to turbulent material flow and rapid flashing of material to atmospheric pressure [64].In steam explosion pre-treatment, the use of sulfuric acid or carbon dioxide decreases time, temperature, and formation of inhibitory products and increases hydrolysis efficiency that ultimately leads to complete removal of hemicellulose. Steam explosion pre-treatment is not that effective for pre-treating soft woods; however, acid catalyst addition during the process is a prerequisite to make the substrate accessible to hydrolytic enzymes. By using steam, targeted temperature can be achieved to process the biomass without the need of excessive dilution. Sudden release of pressure quenches the whole process at the end and also lowers the temperature.

Particulate structure of biomass gets opened by rapid thermal expansion which is used to terminate the reaction. Steam explosion gets affected by certain factors like moisture content, residence time, chip size, and temperature. By two ways optimal hydrolysis and solubilization of hemicellulose can be achieved; either use high temperature and short residence time or low temperature and high residence time. Low energy requirement is a great advantage of steam explosion pre-treatment, whereas in mechanical pre-treatment 70% more energy is required as compared to steam explosion pre-treatment in order to obtain the same, reduced particle size. So far steam explosion pre-treatment with addition of a catalyst is tested and came closest to scaling up at commercial level due to its cost-effectiveness. In Canada, at Iogen demonstration plant, steam explosion pre-treatment is used at a pilot scale. For hardwood and agriculture residues, steam explosion pre-treatment is a very effective pre-treatment process. These effects allow the opening of lignocellulosic structures and influence the enzymatic hydrolysis yield of the material [65]. More than 90% of the initial D-limonene (which has inhibitory effect on yeast) present in the peel waste can be removed from this process.

ii. **Liquid hot water pre-treatment-** This method uses water at elevated temperatures (160 °C–240 °C) under high pressures to keep water in its liquid form in order to promote disintegration and separation of the lignocellulosic matrix [66].

d) **Biological pre-treatment**-
Conventional methods for chemical and physical pre-treatments require expensive reagents, equipment, and high energy. On the other hand it is easy to use biological pre-treatment method which requires live microorganisms for the treatment of lignocellulosic material, and this method is more environment friendly and consumes less energy [11]. There are certain microorganism present in nature that exhibit cellulolytic and hemicellulolytic abilities. White-rot, soft-rot, and brown fungi are known for lignin and hemicellulose removal with a very little effect on cellulose. White rot is able to degrade lignin due to the presence of lignin degrading enzymes like peroxidases and laccases. Carbon and nitrogen sources are involved in the regulation of these degrading enzymes. Cellulose is commonly attacked by brown rot, whereas white and soft rot target both lignin and cellulose contents of plant biomass. Commonly used white-rot fungi species are *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, *Ceriporia lacerata*, *Pycnoporus cinnabarinus*, *Cyathus cinnabarinus*, and *Phanerochaete chrysosporium*. *Basidiomycetes* species including *Bjerkandera adusta*, *Ganoderma resinaceum*, *Trametes versicolor*, *Fomes fomentarius*, *Irpex lacteus*, *Lepista nuda*, and *Phanerochaete chrysosporium* are also tested, and these species showed high efficiency for delignification [67, 2],Also Pre-treatment of wheat straw was studied by Hatakka. [68].

2. **In hydrolysis**- cellulose and hemicellulose are broken down into simple sugars by cellulolytic bacteria's, which can be utilized in the fermentation step to convert it into ethanol [69].Citrus peel also contains two major value-added products: D-limonene and pectin. D-Limonene is widely used in food, cosmetics, and pharmaceutical industries. However, it acts as a microbial growth inhibitor for yeast during the fermentation process and hence it has to be removed prior to fermentation. Pectin is used as thickening agent, gelling agent, and stabilizer in the food industry. Since pectin increases the viscosity of the fermentation medium and makes fermentation troublesome, it has to be either extracted or degraded into galacturonic acid using pectinase enzyme. Thus, the removal and recovery of both D-limonene and pectin from citrus peel are essential for better fermentation (John I. M., 2017). The effects of D-limonene concentration, enzyme loading, and pH on ethanol production from simultaneous saccharification and fermentation (SSF) of citrus peel waste by Saccharomyces cerevisiae were studied at 37 °C [70].

Steps in hydrolysis includes-

a) Isolation of microorganisms**-**Bacteria which have high growth rate as compared to fungi have good potential to be used in cellulase production. However, the application of

bacteria in producing cellulase is not widely used. The cellulolytic property of some bacterial genera such as *Cellulomonas*, *Cellvibrio*, *Pseudomonas* sp, *Bacillus*, and *Micrococcus*, was also reported [24]. Enzyme production is closely controlled in microorganisms and for improving its productivity these controls can be ameliorated. Cellulase yields appear to depend upon a complex relationship involving a variety of factors like inoculums size, pH value, temperature, presence of inducers, medium additives, aeration, growth time, and so forth [71].

b) Collection of samples – The oranges were brought from market, composted soil which was composted with the orange peel waste and it was well aerated with mild exposure to sunlight.

c) Screening and isolating of bacteria -Standard microbiological methods were used to isolate and characterization of the strain. Serial dilution method was followed for isolating the soil microorganisms, Desired dilution will be used which were later pour or spread plated on the CMC agar (carboxymethylcellulose agar) and cellulose agar, incubated for 40,50,55^{0}C for 24hours.The hydrolysing zone were visualized by flooding the plate with 0.1% aqueous solution of congo red or 1% hexa decyl trimethyl ammonium bromide for 15minutes and washed with 1M NaCl.

d) To estimate the cellulolytic activity of the organism, the diameter of the clear zone could be measured on the CMC agar. Besides, a more quantitative assay method was used to determine the cellulose activity of the selected bacterial isolate in liquid medium. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a DNS method. A bacterial isolate with the highest activity was selected for optimization of cellulose production.

e) Bacterial isolation -The bacterial isolates are presumptively isolated based on morphological examination, and biochemical characterization. The parameters investigated with the colony morphology, gram staining, catalase production, VP reaction, indole production, starch hydrolysis and citrate utilization, gelatine hydrolysis. The results will be compared with the bergey's manual of determinative bacteria [72].

f) Enzyme Production Medium- Production medium contained (g/L) glucose 0.5 gm, peptone 0.75 gm, FeSO4 0.01 gm, KH2PO4 0.5 gm, and MgSO4 0.5 gm. Ten millilitres of medium were taken in a 100 mL conical flask. The flasks were sterilized in autoclave at 121^{0}C for 15 min, and after cooling, the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37 ^{0}C in shaker incubator for 24 h.

At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

The optimum condition for *Pseudomonas fluorescens*, *Bacillus subtilus*, *E. coli*, and *Serratia marscens* are pH 7, temp 40^{0}C, 5% carbon source brought the highest cellulase production compared to other % carbon sources at 24 hours of incubation.

According to latest research the best way to isolate the bacteria with cellulase activity from Soil with Optimization were discussed (Fig 1-4) [73].

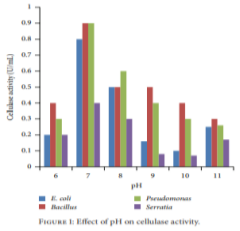


FIGURE 1: Effect of pH on cellulase activity.

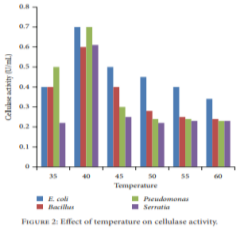


FIGURE 2: Effect of temperature on cellulase activity.

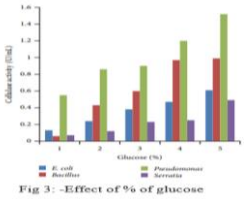


Fig 3: -Effect of % of glucose

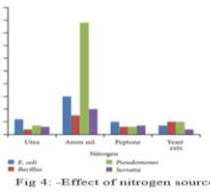
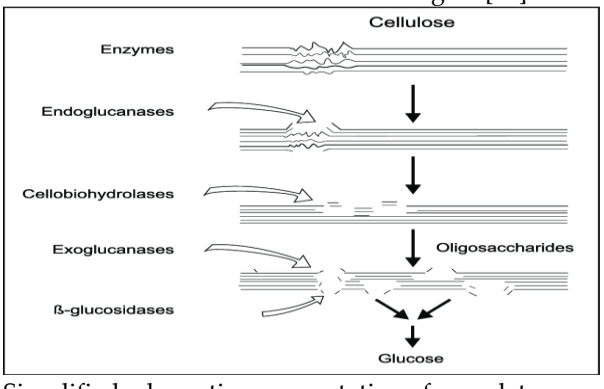


Fig 4: -Effect of nitrogen source

g) Enzyme Assay-Cellulase activity can be measured by the method of Miller. where a reaction mixture composed of 0.2 mL of crude enzyme solution with 1.8 mL of 0.5% carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 7) was incubated at 37^0 C in a shaking water bath for 30 min. The reaction was terminated by adding 3 mL of DNS reagent. The colour was then developed by boiling the mixture for 5 min. OD of samples was measured at 575 nm against a blank containing all the reagents minus the crude enzyme.

h) Enzymatic hydrolysis -microorganisms with cellulase and pectinase were used to hydrolyse orange peel in order to increase the amount of fermentable sugars [70] .



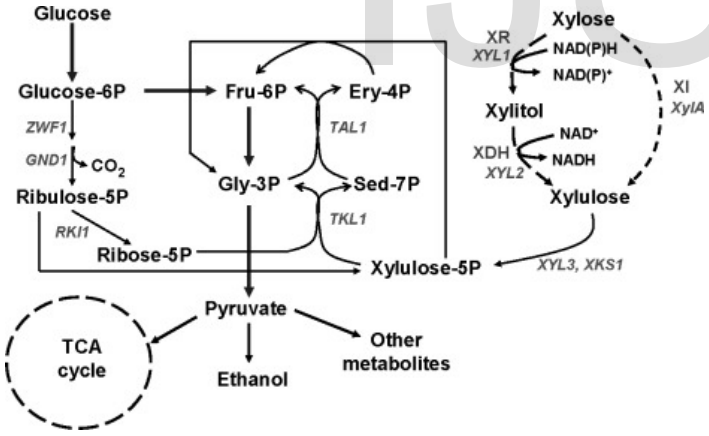
Simplified schematic representation of complete enzymatic hydrolysis of a cellulose by bacteria [74].

3. **Ethanol fermentation-**It is also called alcoholic fermentation, is a biological process which converts sugars such as glucose, fructose, and sucrose into cellular energy, producing ethanol and carbon dioxide as by-products. Yeasts can directly ferment simple sugars into ethanol while other type of feedstocks must

be converted to fermentable sugars before it can be fermented to ethanol [75]. Ethanol fermentation is a Metabolic conversion-In a fermentation process sugar (glucose, fructose or other monosaccharides) is converted to ethanol by microbes (mostly varieties of the yeast Saccharomyces cerevisiae), which are inoculated to the feedstock. The monosaccharides originate either directly from disaccharides, which are broken up through invertase enzymes, or from starch which is hydrolysed with amylase enzymes. In addition to ethanol, water and carbon dioxide are produced also. Ethanol production could be reduced when enzyme loadings were (IU or FPU/g peel dry solids) 0.02 cellulase; and 13 beta-glucosidase, less than 25 pectinases. Ethanol production was greatest when the initial pH of the peel waste was adjusted to 6.0. [76].The glucose-to-ethanol reaction is represented by the equation below:

$C_6H_{12}O_6 + 2\ ADP + 2\ Pi \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ H_2O + 2\ ATP$

Common processes produce a fermentation broth with concentration of 5% - 10% ethanol per volume, as ethanol itself is toxic to the microorganisms. More advanced facilities are able to increase the concentration up to 20% due to the use of adapted and specialized yeasts.



The recovery and concentration of ethanol from the fermentation broth using fractional distillation process which helps in

- Evaporation of ethanol: In this step the first evaporation of ethanol is performed in order to obtain "crude" ethanol with concentration about 45% per volume.
- Rectification: In the rectification step the ethanol concentration is increased to 96% per volume. Here we do repeat fractional distillation to get the ethyl alcohol concentrated.
- Dehydration: By dehydration the remaining azeotropic water is removed in order to obtain a required concentration of 98.7% per mass and water content below 0.3% per mass [77].

Characterization and quantification of bioethanol –

- Gravimetric analysis-The method can be easily incorporated in practice of analytical and control laboratories with no additional material, financial or time costs. Analysis is carried out by the Internal Standard (IS) method. 1-pentanol and 2-pentanol are most commonly used as IS. This method ensures high data reliability [78].
- Uv-vis spectroscopy- A simple and sensitive colorimetric method was developed using 4% solution of sodium dichromate, sulfuric acid and acetate buffer pH 4.3 for determining total ethanol, the methods described do not require expensive equipment's, chemicals. This method is easy to be done in laboratory [79].
- GC-MS method-Ethanol can be measured in using a static headspace gas chromatography coupled mass spectrometry (GC-MS) [80].
- Iodoform test-
  Purity of ethanol test-ethanol reacts with iodine and sodium hydroxide or aqueous sodium carbonate forming a yellow crystalline solid iodoform which is the positive test for ethanol as it has one methyl group linked to the carbon which is attached to the hydroxyl group [81].

Environmental analysis –

Producing bioethanol from the OPW using soil bacteria with cellulolytic activity is of very great interest. Because of its superior environmental impact when compared to Petro-based fuel. On the other hand, the enzyme cost could also be greatly reduced by using naturally available free microbes. According to latest outbreak studies [82]. It is noted that traditional ethanol reduces greenhouse emission by 19-48% compared with gasoline, But the cellulosic biofuels are capable of reducing 100% emission.

Discussion –

Bacteria and fungi both are reported to have cellulase enzyme However, the application of bacteria in producing cellulase is not widely used and recorded in literature till now. The cellulolytic property of some bacterial genera such as *Cellulomonas, Cellvibrio, Pseudomonas* sp, *Bacillus,* and *Micrococcus* was also reported. Enzyme production and activity could be closely monitored and understood with good quality of product recovery [73].

Isolating the soil microorganisms which has cellulase activity could be used for the conversion of cellulose to glucose very efficiently. Generation time of bacteria is approximately 20 mins so even scaling up of bacteria and using them in large may not be a problem [83].

Also, there is a disadvantage of mutation in the microbes which could alter the cellulase activity which could either have beneficial nor detrimental effect. However, the mutation rate is 1 in million which is negligible.

Conclusion –

Many industries and researchers have developed the orange peel waste to bioethanol systems. But yet there are no such breakthroughs into commercial success using the soil microbes in bioethanol production. Many of the limitations are due to technological advancements in cultivation and harvesting as well as in determining the optimal growth conditions for the cellulolytic bacteria.

OPW based bioethanol can potentially solve the constrains imposed on current petroleum-based transportation fuels. Also, there is a need to reduce the operating and process cost to make this bioethanol competitive in market.

However, the orange peel waste-based bioethanol has a potential to be an environmentally friendly, economically feasible alternative to microalgae based, crop-based biofuel and traditional Petro-based fuel.

Therefore, Producing Biofuel from the orange peel waste materials using soil microbes, which is a biotechnological approach in reducing the cost of production of ethanol, environmental pollution and crude oil utilization and dependence on agriculture could be reduced eventually.

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