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Review

Biotransformation of Citrus Waste-I: Production of Biofuel and Valuable Compounds by Fermentation

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Abstract: Citrus is the largest grown fruit crop on the globe with an annual production of ~110–124 million tons. Approximately, 45–55% of the whole fruit post-processing is generally discarded as waste by the food processing industries. The waste is a huge problem to the environment in terms of land and water pollution along with displeasure from aesthetic viewpoint and spread of diseases owing to its huge content of fermentable sugars. The waste can be utilized as a raw material feedstock for producing a number of valuable chemicals and products, such as bioethanol, biogas, bio-oil, organic acids, enzymes, and so on. The production of these chemicals from waste biomass gives an inexpensive alternative to the harsh chemicals used during industrial synthesis processes as well as the possibility of controlling pollution from the waste discarded to the environment. The derived chemicals can be further utilized in the production of industrially important chemicals, as solvents and building blocks of newer chemicals. Furthermore, organic acids, pectin, enzymes, prebiotics, etc., derived from citrus wastes have an edge over their synthetic counterparts in practical applications in the food processing and pharmaceutical industries.

Keywords: citrus waste; bio-waste; biofuel; bioethanol; biogas; biotransformation; fermentation

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1. Introduction

The annual citrus production in the tropical and subtropical regions on the planet exceeds 120 million tons. A significant portion of the fruit ~40–60% is non-edible and discarded as waste. This waste biomass includes peels, pulp and pith residue, and seeds. The pulp and pith residue contain large contents of fermentable sugars, but the peels contain huge amounts of limonene and bioactive molecules which inhibit proper fermentation processes [1–3]. This causes bad odor and pollution to the land, air and water (Figure 1) [4].

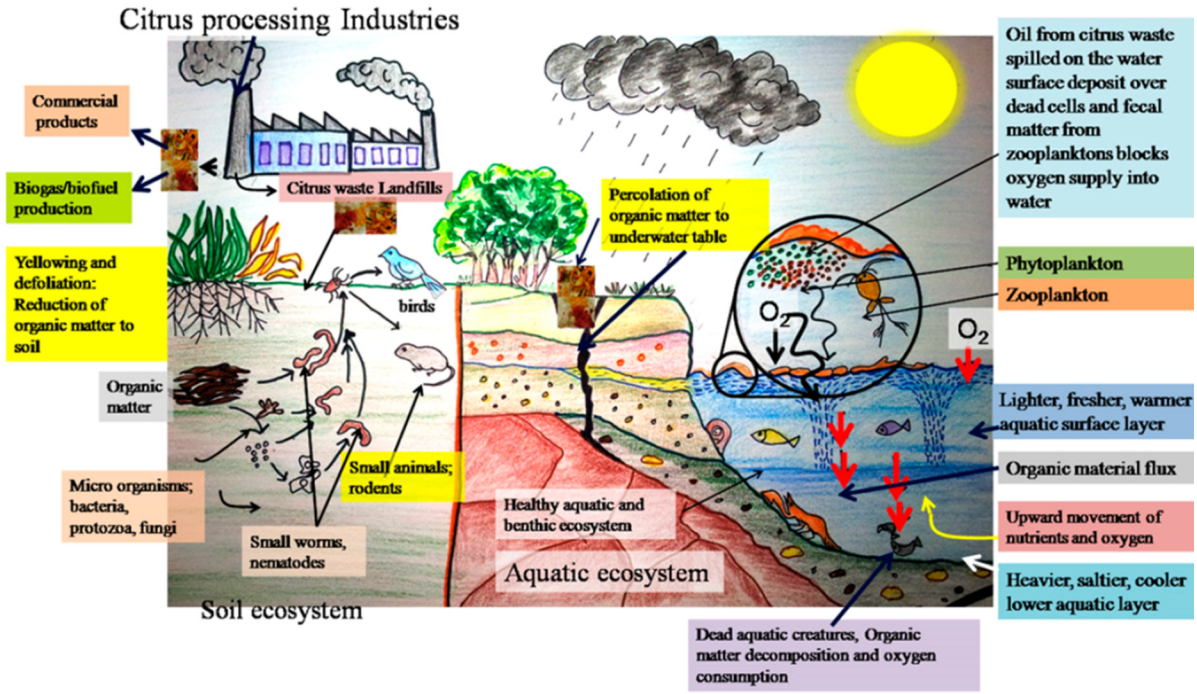


Figure 1. Pollution and deterioration of the environment caused by dumping of citrus waste [4]. Reproduced with permission from Neelima et al., *Foods*, **2019**, pp. 8, 521–579.

Citrus processing wastes contain huge amounts of moisture (75–85%) rendering it difficult to dry. Most of the citrus wastes are dried to transform into cattle feed pellets which requires high consumption of energy. The drying process not only makes the waste remediation expensive but also releases large quantities of volatile organic compounds (VOCs), sulfur dioxide, nitrogen oxides, MeOH, formaldehydes, carbon dioxide, suspended particulate matter, and other compounds or potentially hazardous air pollutants from decomposition of organic compounds present in the biomass in addition to burning of fuel or natural gas utilized for the drying process. It has been reported that the volatile organic compounds in the citrus waste amounts to 9000–18,000 tons/year [5,6]. The environment protection agencies in various countries are now taking stricter measures to abolish air pollution caused by the drying process [7–10].

In past few decades, extensive investigations have been focused on inventing efficient techniques to extract bioactive compounds from natural resources. We have reviewed extensively the various extraction and purification procedures and applications of the bioactive compounds obtained from citrus wastes in our previous publications [1–4,11–13]. The citrus waste (after extraction of bioactive molecules and limonene) can be further utilized to produce ethanol, biogas, fuels, and biosorbents via biotransformation. The latter includes microbial fermentation, and/or physico-chemical processes [14–18]. Citrus biomass, due to its large production worldwide, forms a renewable, sustainable and economic feedstock for obtaining eco-friendly fuel. The carbohydrates and fermentable sugars in the citrus waste are processed to produce biogas and ethanol. The biofuels can reduce carbon dioxide emission by 80% compared to gasoline or petroleum fuel [19–21]. Citrus waste contains ~70% carbohydrates, and capable of producing ~1.2 billion liters of ethanol (or 300 million gallons) on a global scale [22]. In the recent years, ethanol has been explored as an alternative to gasoline fuel, and thus, demand has also increased manifolds. Furthermore, the usage of methyl tertiary butyl ether (MTBE—an additive to the petroleum fuel) is rapidly declining because it releases harmful pollutants upon combustion which are carcinogenic in nature [23]. It can be replaced by ethanol. However, the ethanol addition to gasoline is not advantageous in every case. Ethanol is hygroscopic and may absorb moisture in hot and humid climates prevalent in tropical and subtropical

regions and result in corrosion of automobile internal engine parts. On the other hand, in the countries where cold climate is prevalent, the moisture level in the atmosphere is very low, addition of ethanol to gasoline fuel has not been observed to cause any significant harm to the engine or internal automobile parts. In addition, ethanol is used as disinfectant and organic solvent, in clinical purposes and industrial applications.

Hydrogen is a clean fuel gas and presents a wide range of power options for domestic as well as industrial applications. Currently, hydrogen is produced from non-renewable resources, i.e., fossil fuels by means of steam reforming. In this process, the natural gas reacts with steam under high pressure and at high temperatures in the presence of nickel-based catalyst. Alternatively, hydrogen can also be produced from renewable resources, such as biomass by means of gasification. In the process of gasification, hydrogen is produced via pyrolysis of citrus waste biomass (dried pellets) in an electrically heated furnace. The gas produced from the pyrolysis process is cooled and analyzed for gaseous products and its fuel properties [24]. Volpe et al. carried out torrefaction and slow pyrolysis experiments at 200–650 °C to obtain bio-oil and biochar from lemon and orange-peel wastes by thermal degradation in a horizontal fixed-bed pyrolysis reactor (FBR). Torrefaction of the peel residues in the temperature range 200–325 °C produced fuels with energy densities of 1.56 and 1.58 from lemon peels and orange peels, respectively, and found to be stable at a high temperature of 325 °C. Pyrolysis of the peel residues at 400–650 °C produced high energetic bio-chars and tars [25]. Pyrolysis is a thermo-chemical treatment to the residual biomass to convert it into solid biofuels which is a mix of solid (chars), vapors (tar) and gases. The tar yields of pyrolysis of ~2 g of oven-dried lemon pulp were found to be ~0.4 g of chars [26]. In addition, the citrus waste residues have also been investigated for its promising role in the adsorption of dyes and heavy metals from polluted waters [27].

This article attempts to review the recent progress in the field of biomass energy, biofuels and biosorbent materials for the adsorption of dyes and heavy metals by processing of citrus waste biomass via biotransformation. The article also includes a detailed description of various useful compounds and materials that can be produced commercially from citrus wastes by fermentation. These chemicals find applications in the synthesis and production of various industrially important chemicals of commercial significance. The motivation behind this review is to emphasize the need of the hour to explore the possibilities of harnessing the hidden potential of producing valuable and utilizable products out of citrus bio-waste which, otherwise, goes unnoticed and dumped usually. The review has been presented in two parts under titles "*Biotransformation of Citrus Waste-I: Production of Biofuel and Valuable Compounds by Fermentation*", and "*Biotransformation of Citrus Waste-II: Biosorbent Materials for the Removal of Dyes and Heavy Metals from Polluted Water*". The first part deals with achieving various product from fermentation of the citrus wastes, e.g., biofuels (ethanol, methane and biodiesel) and valuable compounds, viz., organic acids (citric, succinic, pyruvic, lactic, acetic), Vit-C, enzymes, single cell proteins and prebiotics. The second part deals with obtaining adsorbent materials for the adsorption of dyes and heavy metals from waste/polluted water employing thermochemical biotransformation methods.

2. Biofuel: Bio-Ethanol, Biodiesel and Biogas

Grohmann et al. in the 1990s carried out hydrolysis of citrus waste involving enzymes which release sugars. The latter was fermented by *Saccharomyces cerevisiae* (yeast) and *Escherichia coli* (bacteria) to produce ethanol [28,29]. In the later years, a few more reports on enzymatic hydrolysis of citrus wastes to produce ethanol were published [21,30]. Stewart et al. carried out enzymatic hydrolysis using a mixture of enzymes, namely, cellulase, pectinase and β-glucosidase [30]. Prior hydrolysis, limonene is removed in pre-treatment steps from the citrus bio-waste which is antimicrobial in nature and inhibits fermentation process. In subsequent steps, the hydroxylate containing carbohydrates and sugars are subjected to hydrolysis and fermentation to obtain ethanol. There are two main methodologies, viz., simultaneous saccharification and fermentation (SSF), and hydrolysis and

fermentation [21]. The SSF method has the following salient features: (a) it combines enzymatic hydrolysis and microbial fermentation in a single fermenter, facilitating the ethanol production process with a high substrate and enzyme concentration and longer duration of activity by both enzymes as well as microbes; (b) the substrate or the raw material is mainly composed of starchy and cellulosic materials, e.g., sugars (glucose, fructose, sucrose, etc.); (c) the economic method of ethanol production involves less costs of investments; (d) it significantly emphasizes and involves the pre-treatment of raw materials, e.g., enzymatic hydrolysis to breakdown complex organic material prior microbial fermentation; and (e) the affecting parameters are pH, temperature, duration of entire fermentation process, enzyme concentration, substrate concentration, concentration of nitrogen source (ammonium sulfate, urea), potassium and phosphate source (potassium phosphate). The polysaccharides are broken down into sugars and released after the enzyme hydrolysis and subsequently consumed by yeasts and bacteria to produce ethanol.

2.1. Pre-Treatment

The systematic process of ethanol production from citrus biomass requires pre-treatment steps. This includes physical, chemical, physico-chemical and biological pre-treatment. The pre-treatment reduces the total time required for fermentation and production of ethanol. The physical pre-treatments are basically carried out by milling, steam explosion, distillation and drying. Milling is carried out to reduce the particle size of the raw material and simultaneously increase the specific surface area. The reduction of particle size has been observed to improve the hydrolysis yield by 5–25%. The milling process can be carried out in several ways, such as roll milling, hammer milling, colloid milling, ball milling, etc. Post milling, mechanical comminution is often employed which has been observed to improve biodegradability of the lignocellulose biomass. This transformation reduces the degree of polymerization (DP). The results vary according to the type of biomass, duration of operation and type of milling methods chosen. The popping method has been observed to have enhanced the ethanol productivity up to 3.85 g/L h compared to the raw peel waste which is 1.11 g/L h [31]. Apart from this, the physical pre-treatment also includes the hydrothermal treatment at high temperature and pressure. Usually, a combination of pretreatments is employed. On the other hand, chemical pre-treatment includes the action of acids or alkalis on the citrus biomass which selectively carry out breakdown of biomass components. Strong acids facilitate removal of hemicellulose and lignin, whereas the alkalis neutralize the pH. Chemical treatment produces inhibitor products which retard fermentation process, hence requires removal of the same [18,21,32–34].

Steam explosion is generally carried out at 160–260 °C for 5–20 min in a pressure reactor followed by pressure release. In this process, pressurized steam is introduced to the citrus peel biomass in a reactor and then vented out after a fixed reaction time which allows a quick reduction of pressure in the reactor [32]. Steam at high temperature, sterilizes the biomass and ruptures thoroughly the plant cell walls made of complex polysaccharides into simpler and digestible compounds. The latter facilitates the enzymatic action. The steam explosion treatment is an energy intensive process which helps in the breakdown of the complex structural polysaccharides into simpler 5-and 6-carbon sugars. The pre-treatment facilitates absorption of acid and enzymes to act on the cellulosic molecules. The affecting factors are particle size, surface area and particle morphology. It has been demonstrated that the reduction of particle size in the sample undergoing hydrolysis reduces the time taken for the hydrolysis from days to hours. Choi et al. carried out popping pre-treatment in which homogenized peel waste was treated in a popping reactor (a 300 m long horizontal cylinder with 3 liters of volume capacity) at 150 °C under a pressure of 15 kgf/cm for 10 min without using any chemicals. They observed a reduction of *d*-limonene from 0.21% to 0.01% which facilitates fermentation process smoothly. Popping treatment helps in rupturing the peel cell walls and reducing the particle size below 1 mm. Although, it did not affect the sugar concentration in the hydroxylate significantly, but improved the ethanol production up to 46.2 g/L compared with 39.8 g/L from raw peel waste without

treatment [34]. Furthermore, the steam explosion also enables the recovery of limonene up to 94.3%. Post steam-treatment, the biomass is separated out. This step is crucial as the limonene is antimicrobial in nature and inhibits the microbial action which results into poor digestion of the biomass during fermentation [33,35–37]. The steam explosion process allows rapid decomposition of the biomass and facilitates better cellulose hydrolysis and release of sugars [31,38]. The steam explosion process can be carried out in two ways; (1) High temperature and short hold time (270 °C, 1 min), and (2) low temperature and long hold time (190 °C, 10 min). Furthermore, the method can be made more effective by addition of acid which can act as a catalyst. On the other hand, the disadvantages of this method are: (a) loss of xylan fraction in the biomass, (b) formation of species which are inhibitory to the fermentation process, and (c) inhomogeneous disruption of cell walls leading to incomplete digestion, although this method facilitates the removal/recovery of *d*-limonene (reduction from 0.8 wt. % to less than 0.1 wt. %) [5,31].

2.2. Esential Oils: Removal and Recovery of Limonene

Citrus peels are rich in essential oils. Limonene (91–95%) is the major component of the essential oils followed by pinolene (1.83–2.61%), *n*-octanol (1.50–1.64%), myrcene (1.3%), α-pinene (0.28–0.5%), linalool (0.39%), β-pinene (0.38–1.05%), γ-terpinene (0.41–1.09%), camphene (0.27–0.35%), decanal (0.11–0.35%), and so on. Besides, there are manty minor to trace amounts of components, e.g., geraniol, geranial, neral, terpinene-4-ol, nerol, δ-elemene, 3-carene, isopulgol, δ-cardinene, sabine, α-phellandrene, 1,4-cineole, trans-β-ocimene, *n*-octanol, *cis*-epoxylimonene, perillaldehyde, β-caryophyllene, germacrene D and β-myrcene are also present in citrus essential oils. The antimicrobial effects of minor components of the citrus essential oils on the fermentation process has not been extensively researched so far [3,39–41].

Removal and/or recovery of limonene are considered to be one of the most important steps prior fermentation of biomass to produce biogas/biofuel. The main pretreatment methods reported for the removal of limonene are aeration, and biological treatment. On the other hand, the main pre-treatment methods for the recovery of limonene are expression or 'cold pressing', dry distillation, filtration, sorption, centrifugation, steam distillation, steam explosion and liquid extraction with organic solvents. Usually, the yield is in the form of watery emulsion, and therefore, subjected to centrifugation to separate oils from aqueous phase. The cold pressing technique does not disturb the chemical composition of the essential oils, whereas in other techniques, the operation at an elevated temperature causes chemical modification in the components, particularly volatile organic compounds [3,4]. Prolonged exposure to high operational temperatures may also cause disappearance of volatile organic compounds. The two processes, viz., removal and recovery have fundamental differences as the latter can yield useful outcomes in economic terms [4,31,42,43]. Choi et al. developed a column containing sorbent material to separate *d*- limonene from the enzyme hydroxylate. The sorbent material is made of raw cotton and active carbon which exhibits high oleophilic and hydrophobic properties, and above all, biodegradable in nature. The sorbent materials are generally classified into three major categories; natural materials, treated cellulose or petrochemical polymers. Polymers such as polypropylene, polyethylene and polyurethane are non-biodegradable and create pollution. Sorbent material made of cotton (high absorbing properties) and activated carbon (high porosity) adsorb significant amounts of chemicals [34,41]. Chávez-González et al. reported on enzymatic pretreatments using cellulase on the citrus peel waste (orange, lemon, and grapefruit) and achieved increased yields of essential oils extracted by hydro-distillation [40].

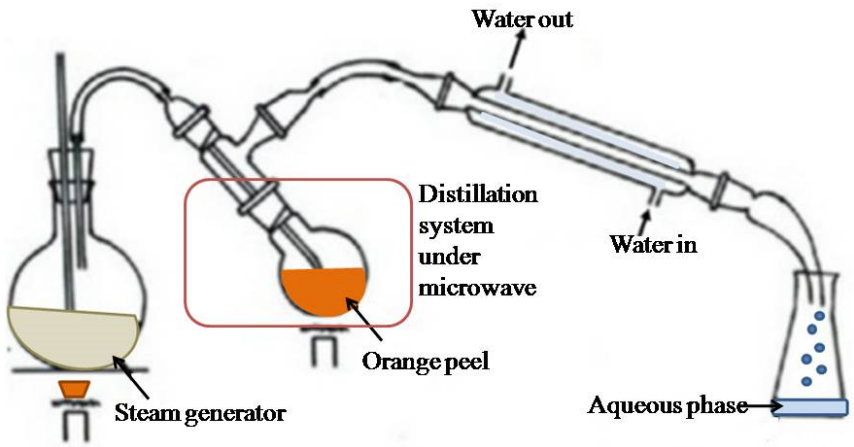
Biological treatment is primarily carried out by the activity of fungi, e.g., selected strains of *Sporotrichum*, *Aspergillus*, *Fusarium* and *Penicillium*, or enzymes obtained from fungal strains, e.g., *Aspergillus* and *Penicillium* [42,43]. The latter resulted in reduction of limonene concentration in the treated biomass by 55%. The treatment of the citrus peel biomass with *P. digitatum* results in biodegradation of limonene into α-terpeneol [35,44–47].

A bioconversion efficiency of >90% was recorded by Badee et al. Besides α-terpeneol, carveol and carvone are also produced and impart anti-microbial activities, therefore, impedes the natural fermentation process [48]. Some of the compounds present in essential oils show stronger antimicrobial properties compared with limonene [37,46,49]. α-terpeniol exhibits antimicrobial properties ~1000 to 5000 times greater than limonene on certain bacterial strains, e.g., *E. coli* and *S. aureus* [50–52]. Apart from inhibiting the natural fermentation process by the microbes, limonene also causes accumulation of fatty acids. The latter leads to system failure [53].

Ethanolic extraction of limonene is carried out using an aqueous mixture of 70% ethanol in water by volume and adding to the citrus peel waste biomass in a ratio 1:10 (peel: solvent). The mixture is stirred for an hour or two, either at room temperature or an elevated temperature of 40 °C and the solvent phase is separated which contains dissolved limonene. Apart from limonene, the citrus biomass also contains adequate amounts of bioactive compounds that are generally extracted using steam distillation methods. Steam distillation helps in the recovery of limonene along with other major and minor components present in the citrus essential oils. According to the available records, ethanol extraction of limonene has been found to be most effective method followed by steam distillation or biological treatment. In recent years, modern methods, such as pulsed electric field, subcritical water extraction (water as an extractant, at 100 °C–374 °C and high pressure ~22.4 MPa); instantaneous controlled pressure drop process (DIC), microwave assisted extraction, vacuum microwave hydro-distillation, solvent free microwave extraction, microwave steam distillation, microwave hydro-diffusion and gravity, supercritical fluid extraction and pressure drop technique have been introduced and found to be effectively yielding greater results. These techniques are quick in yielding, helping to preserve the fragrance quality of the yields and consume less energy during the operation. Supercritical fluid extraction method uses CO_2 above its critical temperature and pressure (30 °C–40 °C; 300 MPa) as fluid for extraction. This technique consumes less solvent and leaves no residue in the extract as it can be evaporated out. In addition, it facilitates rapid extraction, easy concentration with high selectivity. Ultrasound assisted extraction techniques uses very high frequency sound waves which create cavitation effects. The latter accelerates heat and mass transfer to disrupt the cell walls to release the contents. The method yields quick results with high reproducibility at low solvent consumption [14,39,54–60].

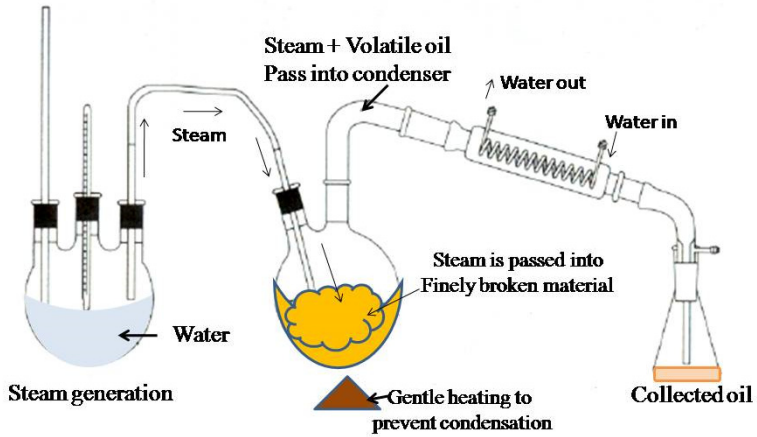
Post removal of limonene from the citrus waste biomass, the fermentation process is carried out using microorganisms to produce ethanol. This is classified under biological treatment. In this process, the enzymes extracted from fungal strains, namely *Aspergillus* and *Penicillium* are employed to promote anaerobic digestion [35,37]. A summary of pre-treatment methodologies commonly employed in the extraction of limonene and other bioactive compounds from the citrus processing waste have been illustrated in Figure 2.

Microwave Steam Distillation (MSD)



- Used to recover the essential oils
- The citrus peel waste is placed in a microwave reactor and internal heating burst the cell walls
- Microwave irradiation power: 135 W–445 W; 5–10 min
- Extraction process is followed by azeotropic distillation

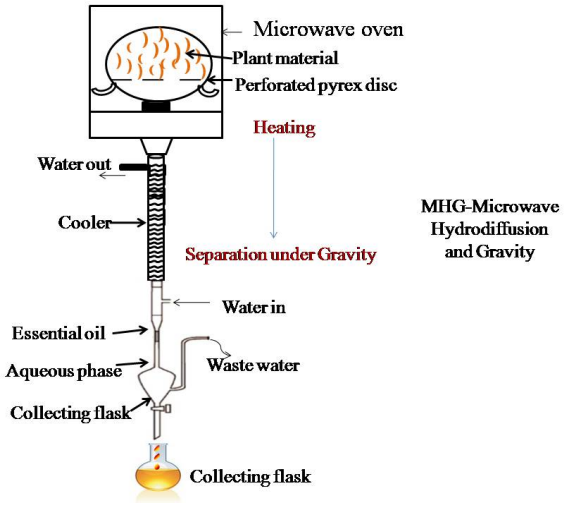
Steam Distillation



- Performed before dehydration of plant materials
- 3 types of HD-water distillation, water and steam distillation and direct steam distillation
- The plant materials are packed in a still compartment and water is added in sufficient amount and then brought to boil or direct steam is injected into the plant sample
- Hot water and steam rupture plant cells and release bioactive compounds
- Indirect cooling condenses the vapor mixture containing oil which flows from condenser to a separator, where oil and bioactive compounds separate automatically from the water

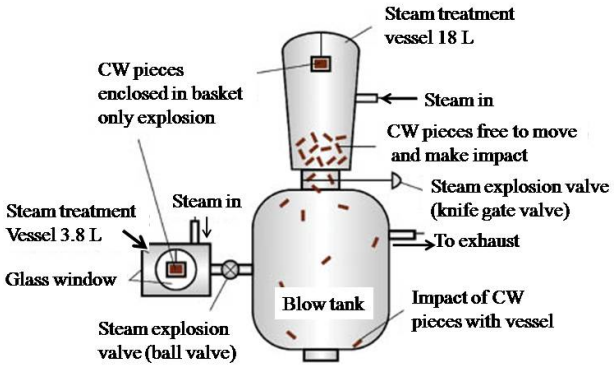
Figure 2. *Cont.*

Microwave hydro-diffusion and gravity (MHG)



- No solvent used
- Microwave power 500 W for15 min
- Combination of microwave heating and gravity working at atmospheric pressure
- The plant material is directly placed in a microwave reactor without any added solvent or water
- Heating of the water in situ distends the plant cells and rupture of the glands and cell receptacles
- Heating frees molecules of interest together with in-situ water, i.e., hydro-diffusion
- Extract drops by gravity out of the microwave reactor through the perforated Pyrex disc

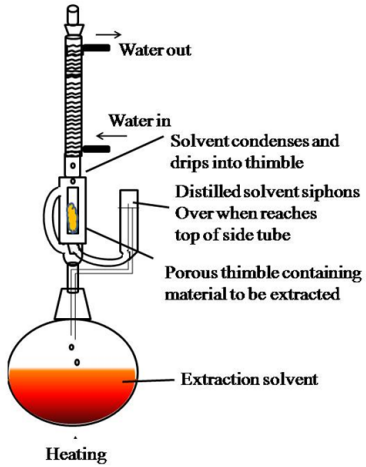
Steam Explosion



- Pressurized steam is introduced to the citrus peel biomass in a reactor
- Carried out by two methods: (a) High temperature and short hold-time (270 °C, 1 min); (b) moderate temperature and long hold time (190 °C, 10 min)
- The pressure is vented after a fixed interval of time
- Rapid decomposition of the biomass
- Affecting factors: substrate particle size, residence time, temperature, moisture content

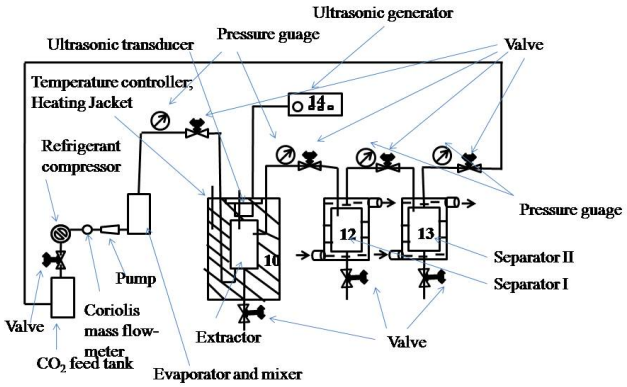
Figure 2. *Cont.*

Soxhlet Extraction



- A small amount of dry sample is placed in a thimble, and placed in a distillation flask which contains the solvent of particular interest
- After reaching to an overflow level, the solution is aspirated by a siphon. Siphon unloads the solution back into the distillation flask
- The solution carries extracted solutes into the bulk liquid and the solute remained in the distillation flask and the solvent passes back
- The process runs repeatedly until the extraction is complete; water is used as solvent,
- Temperature 100° C; duration 6–10 h

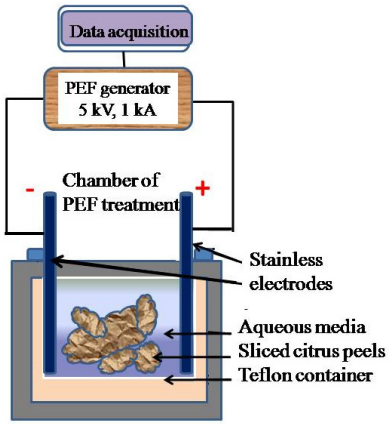
Supercritical Fluid Extraction



- The system consists of a tank of mobile phase, CO_2, a pump to pressurize the gas, co-solvent vessel and pump, an oven containing extraction vessel, a controller to maintain the high pressure inside the system and a trapping vessel; CO_2 (at 31 °C and pressure at 100 and 450 bar)
- Ultrasonic techniques can enhance SC-CO_2 extraction
- Both extraction method applied together result in higher yield compared to individual process
- Ultrasonic power outputs 0–400 W; enhanced by ultrasound resistant pressure of 35 MP at temperature 55 °C

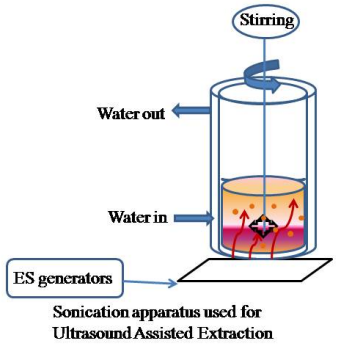
Figure 2. *Cont.*

Pulsed Electric Field Assisted Extraction (PFE)



- In this method the living cells are suspended in an electric field and the electric potential passes through the membrane of the cell based on the dipole nature of the membrane molecules
- Electric potential separates the molecules according to their charge in the cell membrane
- After exceeding a critical value of ~1 V of transmembrane potential, repulsion occurs between the charge carrying molecules which form pores in weak areas of the membrane and causes drastic increase of permeability and release of contents

Ultrasound Assisted Extraction



- Waves pass through the medium by creating compression and expansion and produces cavitation
- A large amount of energy can be produced from the conversion of kinetic energy of motion into heating the contents at high temperature
- The extraction mechanism involves two main types of physical phenomena, (a) the diffusion across the cell wall, and (b) rinsing the contents of cell after breaking the walls
- Sound wave frequency in range of 20 to 100 MHz

Figure 2. Pretreatment techniques employed for the extraction of limonene and other bioactive compounds from citrus waste [4]. Reproduced with permission from Neelima et al., *Foods*, **2019**, pp. 8, 521–579.

2.3. Hydrolysis

The complex structural polysaccharides are required to be converted into simple sugar monomers prior fermentation process. The breakdown process is successfully carried out via hydrolysis; either separately in different reactor tanks or simultaneously with fermentation process in the same reactor tank. The main hydrolysis processes are, (a) chemical hydrolysis, and (b) enzyme hydrolysis. Other methods are microwave irradiation or gamma-ray or electron beam irradiation, which have been employed for breakdown of complex structural polysaccharides. However, these methods have limited involvement as these are energy consuming, difficult to handle and not economical. The lignocellulose upon hydrolysis generates hexoses and pentoses: cellulose upon hydrolysis generates hexoses (glucose), and hemicellulose upon hydrolysis generates mannose, galactose, glucose, acetic acid and xylose. Xylose can be further converted to xylitol via bioconversion which is marketed as a functional sweetener [61,62]. On the other hand, xylan degradation generates a number of products, viz., acetic acid, 2-furfuraldehyde, formic acid, hydroxy-1-butanone, propionic acid and hydroxy-1-propanone. Furthermore, upon elevation of temperature and pressure, xylose degrades to generate furfural and hexose produces 5-hydroxymethyl furfural (HMF). Uncontrolled hydrolysis has been observed to generate aldehyde and furfural which reduce sugar yields and poisoning of the fermentation medium [63,64].

(a) *Chemical hydrolysis*: Chemical hydrolysis can be an integral part of both the processes, namely pretreatment and hydrolysis assisted fermentation. It can be carried out by using either dilute or concentrated acids, namely, hydrochloric acid or sulfuric acid. Sulfuric acid at a pH of 2.0 is generally considered to be highly effective for hydrolysis of citrus waste biomass in terms of less acid consumption and lower destruction of fructose [65]. Increasing the acid concentration from 0.05% to 1.0%, (w/v) has been observed to increase the release of sugars into the slurry. However, there is also observed an increase in the amounts of HMF, acetic acid and phenolics which are inhibitory to the fermentation process. Hence, the chemical hydrolysis process is proposed to be carried out in two stages with dilute acid (H_2SO_4, 0.5% w/v in the second stage) to improve the yields. In addition, longer duration of hydrolysis than the optimized duration has also been witnessed with formation of inhibitory HMF in the medium [31].

(b) *Enzymatic hydrolysis*: This is the most favored mode of hydrolysis for the citrus processing waste in order to produce ethanol. Enzyme hydrolysis is also known as enzymatic saccharification in which the cellulosic and hemicellulose fraction in the biomass is digested by enzymes to generate pentose and hexose sugars and utilized by microbes in the fermentation process. Researchers have employed cocktails of enzymes in different proportions and recorded the effects of different amounts of loading. The commonly employed enzymes for hydrolysis process are cellulase (1.9 mg of protein/g of total sugar), pectinase (0.8 mg of protein/g of total sugar), and β-glucosidase (1.6 mg of protein/g of total sugar). Cellulase breakdown cellulose and the commonly employed cellulase enzymes are, endo-1,4-β-glucanase (EC 3.2.1.4), exo-1,4-β-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). Pectinase breakdown pectic substances present in the cell wall; decrease the viscosity of the medium and soften the tissues. The commonly employed pectinase enzymes are polygalacturonase (EC 3.2.1.15), pectin lyase (EC 4.2.2.10), and pectin esterase (EC 3.1.1.11). Enzymatic hydrolysis is an environment-friendly process and does not generate any toxic products as a result of degradation. The moderate conditions for carrying out enzymatic hydrolysis are; temperature 45–50 °C, pH of 4.8–5.0 for 24 h. When xylanase was employed along with cellulase, pectinase and β-glucosidase for the hydrolysis of mandarin peel waste, the hydrolysis generated galacturonic acid, rhamnose, arabinose, glucose, galactose, xylose, fructose and sucrose. The pectinolytic and xylanolytic activities are facilitated by *Aspergillus* sp., and cellulolytic activities are facilitated by *Trichoderma* sp. The enzymatic hydrolysis is pH sensitive. To obtain best results, the mixture is supplemented with acetate (sodium acetate) buffer of

pH 4.8. As the enzymes and nutritional supplements are added, the pH of the mixture becomes stable between 4.8 and 5.2 and the constituents are incubated at 35 °C with constant rotation. Since, commercial enzymes are expensive; the overall production of ethanol becomes expensive too. One of the successful resolutions in this case has been in-house production of these enzymes using microorganisms [31,34,41,66–69].

As a result of hydrolysis, production of reducing sugars and a drop in the pH (from 4.8 to 4.3) takes place. The latter is caused by the release of D-galacturonic acid from pectin in the cell walls. The pK_a of D-galacturonic acid is 3.51 and its continuous release causes a drop in the pH value of the hydroxylate medium down to 3.3–3.5. This substantial drop in the pH from 4.8 to 3.3 inhibits the enzymatic activity and the overall hydrolysis process. Therefore, to stabilize the system again to the optimal pH, calcium carbonate (solid) in the quantity slightly less than the equimolar amounts to galacturonic acid, is added to the hydrolysis mixture. However, addition of $CaCO_3$ may reduce the sugar yield by ~10–15% compared with the one where it is not added. This is believed to be due to the formation of cross-linking and precipitation of pectin by calcium ions rendering the peel substrate more resistant to hydrolysis by the enzymes. The peel oil components were found to be adhered to residual insoluble solids post enzymatic hydrolysis and can be separated out by filtration [14,28,31,70].

Post enzymatic hydrolysis the mixture now contains high levels of galacturonic acid and arabinose which are not fermentable to ethanol by the action of yeasts. In such, a case *E. coli* KO11, an ethanologenic recombinant strain of the bacterium, is employed to carry out the fermentation of galacturonic acid which results in the production of equimolar amounts of acetate and ethanol along with CO_2. Fermentation by recombinant *E. coli* KO11 has been observed to increase the ethanol yields by 25–35% compared with fermentation by yeast. The yeast, *S. cerevisiae* could ferment six carbon sugars, glucose and fructose but cannot utilize five carbon sugars, arabinose, xylose or galacturonic acid in the peel hydroxylates. The galacturonic acid is the main constituent of pectin and the amount of the release of the acid during hydrolysis process indicates the extent of pectin hydrolysis [28,71,72].

2.4. Fermentation

2.4.1. Ethanol

The fermentation process is carried out by several methods, viz., (a) batch, (b) fed-batch, (c) continuous process, (d) submerged fermentation, (e) continuous immobilized fermentation, depending upon the composition of lignocellulosic hydroxylates, behavior of the microorganisms, and economical aspects. The fermentation process may be carried out with and without in association of enzymatic hydrolysis. In this aspect, the process can be categorized into, (f) separate hydrolysis and fermentation (SHF) and (g) simultaneous saccharification and fermentation SSF [14,31].

(a) *Batch Fermentation*: In the batch process, the reactor vessel is supplied with a limited-set amount of nutrients and microbial inoculation at the initial stage or at zero time and the fermentation process is allowed to run under a controlled environment of temperature, pH and pressure until maximum yield of end products (concentration) is achieved. During the entire process, no additional nutrients are added, and hence it is a closed system. At the end, all the nutrients are consumed and it is possible to characterize the microbial strains and optimize the nutrient medium to develop a rapid mechanism. However, the product yield from the biomass remains limited. In this process, the microbes have limited time to remain in the exponential growth phase because the carbon sources (biomass and the nutrients) and oxygen supply act as limiting factors. The process is usually facilitated with increasing the stirring speed, the oxygen gas flow into the medium. At the end, the medium is collected and proceeds to further processing to obtain the desired form of the product. The main advantages of this method are; (i) short duration of operation (ii) reduced or limited chances of contamination as no biomass substrate or nutrients are added during the process, (iii) ease of separation of batch material for traceability, and (iv) convenient

to handle and operate. On the other hand, the disadvantages are (i) the end product is mixed with substrate biomass, nutrients cell debris and metabolic toxins, and (ii) productive time is short, resulting in low yields [14,31].

(b) *Fed-batch fermentation*: In this method, a controlled addition of substrate and nutrients is carried out at desired intervals during the entire fermentation process. The system is partly open unlike the batch system which is completely closed. The substrate is pumped from the supply vessel to the culture reactor through a silicone tube attached to it. The fed-batch process allows specific growth conditions to the microorganisms to a prolonged duration and an exponential growth curve is maintained for a relatively longer duration. Eventually, an increased feed rate is required. Although, the fed-batch method allows a range of control strategies and extended operational durations, the fermentation process faces inhibition because of the formation of toxic by-products. The fed-batch method is also known as semi-continuous process. The main advantage of the fed-batch method is that it allows an increase in the concentration of viable microbial colonies, product accumulation and sufficient culture life time [31,73].

(c) *Continuous culture fermentation*: In this method, the substrate and nutrients are continuously fed to the reactor after a batch growth phase attains an equilibrium stage or a steady-state and products are removed. The removed medium containing desired products as well as by-products is utilized to harvest microbial cells and residues besides ethanol. Hence, it is usually run with a programed inflow and out flow mechanism adjusted with intervals less than doubling time of the microorganisms employed. Although, this method is an advantage to the fed-batch mechanism, however, the prolonged cultivation period with repeated feeding of fresh substrate to the medium in the reactor increases the risk of contamination. Furthermore, it is difficult to (i) maintain a constant microbial population density inside the reactor medium over sustained duration, (ii) carry out separation of products from the batches and control for changes in the genetic material/mutation of the microorganism [31,74,75].

(d) *Separate hydrolysis and fermentation (SSF)*: In this method, the enzymatic hydrolysis is carried out separately from the microbial fermentation process. Here, the medium from hydrolysis reactor is transferred to the fermentation tank and the ethanol is distilled off leaving behind unconverted sugars and microbial cell mass. The separation of the two fundamental processes, viz., enzymatic hydrolysis (at 45 °C–50 °C) and fermentation (at 30 °C), facilitates optimum favorable conditions for both adequately. On the other hand, the inhibitory effect caused by the sugars (e.g., glucose), produced from hydrolysis, to the enzymatic actions of cellulase and β-glucosidase enzymes retards completion of the hydrolysis process and might require higher or additional loadings of enzymes to the reactor to achieve good yields of fermentable sugars. Choi et al. have observed an ethanol yield of 90.6% from pretreated mandarin peels by SHF process. They reported an ethanol concentration of 46.2 g/L with a productivity of 3.85 g/L h [34].

(e) *Simultaneous saccharification and fermentation (SSF)*: In this process, both the enzymatic hydrolysis and fermentation processes are carried out in the same tank. Since, the products released from hydrolysis, viz., glucose, xylose, and cellobiose by the action of cellulase, xylanase, etc., cause inhibition to further hydrolysis and saccharification process, therefore, these are required to be consumed by the microbial fermentation process. Due to the combined processes running simultaneously, the system requires a relatively shorter duration of operation, lower enzyme requirement, facilitates higher product yields, effective conversion of fermentable sugars to ethanol and eventually regulating the inhibitory effects of the sugars on the enzymatic action. In addition, there is a lower requirement of sterile conditions in this process as the sugars produced during the hydrolysis by enzymes are quickly consumed by the microbes for producing ethanol. Boluda-Aguilar et al. investigated the yield capacities of the two processes, and reported that SSF resulted in higher yields (50 L/1000 kg of citrus peels) compared to the SHF (50 L/1000 kg of citrus peels). The residue remaining after the

distillation of ethanol have been found to be lower in SSF than SHF which is converted to citrus pulp pellet or animal feed and manure. Most importantly, the production of ethanol by either of the fermentation processes also greatly depends upon pre-treatment. The hydrolysis of citrus cellulosic biomass under SSF to produce ethanol has been reported to be the least expensive method. Moreover, the by-products of ethanol, viz., syngas ($CO+H_2$) can also be converted back to ethanol [5,31,66,67].

(f) *Continuous immobilized fermentation*: In this method, a continuous fermentation process is carried out using immobilized microorganisms. The immobilized microbes can be recovered post-fermentation process and reused again. The microbes are immobilized by housing them inside a column. Choi et al. developed an Immobilized Cell Reactor (ICR) by housing immobilized *Saccharomyces cerevisiae* cells, packed up to 70% of the column volume (column dimension 25 cm long with an internal diameter of 2.1 cm). The fermentation medium was introduced to the ICR from limonene removal column (LRC) and fermentation was run for 10 days at 30 °C in an incubator. The process was reported to yield a 12 folds higher yields of ethanol [31,41].

2.4.2. Biogas

Biogas is largely methane and carbon dioxide combined with small amounts of N_2, O_2, H_2 and H_2S. It is naturally produced in marsh lands, swamps, rumen of ruminant animals, rice fields and garbage landfills, where oxygen is depleting in the surrounding environment. Artificially, it can be produced by anaerobic digestion in a fermentation tank. The anaerobic digestion comprises two main steps, namely, (a) acidogenesis, and (b) methanogenesis. The hydrolysis of citrus biomass is carried out by four major processes, viz., (i) acid hydrolysis, in which the biomass is treated with dilute and concentrated acid; (ii) enzymatic saccharification, where the citrus biomass post limonene removal is treated with enzymes extracted from fungal strains; (iii) fermentation in which the hydrolyzed biomass is acted upon by microbes; and (iv) SSF/SHF. The enzymatic hydrolysis or saccharification is carried out by employing a number of enzymes, mainly lipase, protease, amylase, cellulase, hemicellulose, etc. [76,77]. This process continues for few hours to several weeks until completion depending upon the nature and complexity of the substrates. For example, simple organic compounds such as carbohydrates and simpler polysaccharides require less time (few hours to a day) for completion of hydrolysis, whereas complex molecules such as proteins and lipids as well as cellulose and hemicellulose take weeks to complete the hydrolysis process. The reason is the bonds in carbohydrates are weak with less bond dissociation energy. When acted upon by acidogenic bacteria, the breakdown products are acetate, hydrogen and CO_2 along with intermediate products such as alcohols, volatile fatty acids. These are consumed by acetogenic bacteria to produce acetate, H_2 and CO_2. In this process, approximately, 70% of the total carbon present in the biomass in converted into acetate and rest of the 30% is converted into CO_2 [78]. Under limited or no oxygen in the environment, i.e., strict anaerobic conditions, methane gas is produced by the bacteria. Acetogenic bacteria which produce acetate also produce hydrogen. These live in symbiotic association with methanogenic bacteria under a low partial pressure of hydrogen ($<10^{-5}$). Methanogenic bacteria produce methane by assimilating hydrogen and hence maintain a lower partial pressure in the surrounding region. On the contrary, there is another category of bacteria, namely, homoacetogenic microbes present in the biomass substrate which convert acetate from hydrogen and carbon dioxide. When the partial pressure of hydrogen is high ($>10^{-6}$ bar), hydrogenotropic bacteria present in the biomass substrate produce methane from H_2 and CO_2. Thus, in a biogas reactor, collective and symbiotic action of microbes produce methane as main biogas component [78,79].

The methane producing microbes are sensitive to several parameters, such as pH, temperature (20–50 °C), substrate concentration, and so on. The peak production of methane has been reported to take place in the duration between 5 to 25 days. In one such laboratory experiments carried out by Kaparaju et al. where thermophilic digestion of orange wastes received from food processing industries to produce 0.49 m^3 of biogas per

kg of volatile solid. The slurry is treated with $NaHCO_3$ and added back to the substrate. This procedure maintains the pH of the substrate in the reactor environment and facilitates microbial action. They carried out another experiment which employed semi-continuous system. A loading rate of ~2.8 kg per cubic meters of organic waste material per day into the reactor with a hydraulic retention time of 26 days along with pH adjustment regulated by adding $NaHCO_3$ and NaOH. The experiment yielded 0.60 m^3 of methane per kilogram of biomass feed from the anaerobic digestion [53]. On the contrary, the biomass without limonene removal produced 0.10 m^3 per kilogram of volatile solid biomass. The substrate is termed as 'volatile solid' here. The reduced production of biogas is because of limonene's inhibitory activity on microbial growth and action. When steam explosion step was introduced to the substrate pre-treatment at 150 °C for 20 min, it was observed an increase of methane production by 426% compared to the experiment where the citrus biomass was not given pre-treatment. Under optimized and near ideal conditions, a quantity of 100 kg of citrus waste feedstock has been observed to produced 40 L of ethanol, 45 m^3 of methane, 9 liters of limonene and 39 kg of pectin [32].

Biogas reactors carry out biomass digestion by two basic methods, viz., (a) batch digestion method, and (b) semi-continuous digestion method. In batch digestion method, the temperature inside the reactor is maintained at 25–50 °C to facilitate an optimal growth and functioning of the microorganisms. The digestion process inside the reactor is usually ensured by anaerobic fermentation which is brought upon by flushing gas with nitrogen and carbon dioxide (N_2–80%, and CO_2–20%). On the other hand, the semi-continuous digestion method utilizes the stillage and solid biomass residue obtained after the pre-treatment. This method of digestion requires continuous stirring of the material inside the reactor tank and periodic invigilation. Both the methods produce ethanol and methane (biogas) as a result. Citrus waste contains adequate amounts of micronutrients, such as nickel, iron, zinc, cobalt and magnesium which are important for the growth and functioning of the methanogenic bacteria [32,80].

One of the major challenges in the process of microbial digestion of citrus biomass to produce ethanol and biogas is the energy in terms of electricity required for pre-treatments, viz., mechanical churning of the raw materials, steam explosion, distillation, removal of limonene, drying, etc., and procurement of enzymes, which are expensive. These costs add up to make the entire cost of production of ethanol expensive. In this regard, a relatively newer approach which involves dilute acid hydrolysis of citrus waste biomass has been introduced [81]. However, this method is limited up to demonstration stages or laboratory scale experiments only, and still to be explored at industrial scale. Additionally, it has been found to yield lesser amounts of carbohydrates and simple sugars from the biomass digestion. An alternative method to anaerobic digestion, simultaneous saccharification and fermentation has been observed to yield up to 76–94% of ethanol under 48 h of operation, simultaneously controlling both, pollution and the costs. The resultant products, biogas contains 65–70% of methane [71,82]. Removal of *d*-limonene from the citrus waste biomass prior digestion process has been observed to yield higher quantities of methane. Another experiment on systematic biodegradation of citrus waste biomass using microbial strains, viz., *Phanerochaete chrysosporium* ATCC 20,696 and *Aspergillus niger* CCTCC 206,113 have been reported to yield biogas production of 308.85 mL per gram of volatile solid with methane content of 176.05 mL per gram of volatile solid [17]. The yield of methane from the anaerobic batch digestion of citrus biomass with and without pre-treatment is presented in the Figure 3.

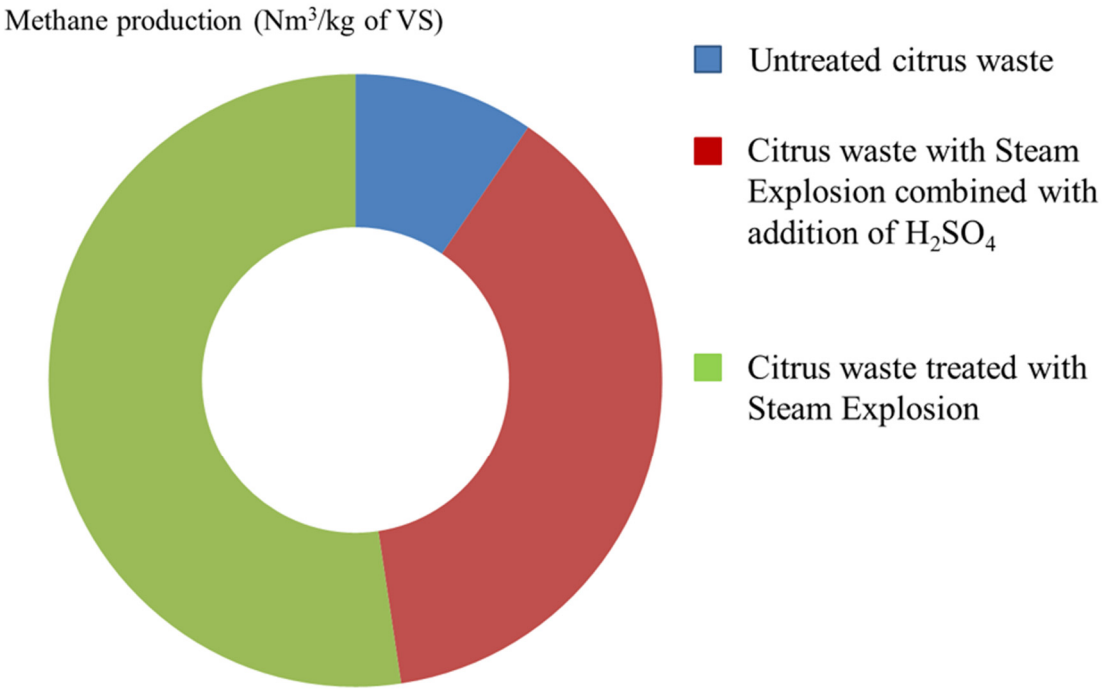


Figure 3. Methane production from citrus waste and comparison of results obtained from biomass untreated, and upon pre-treatment; steam-explosion (at 150 °C for 20 min), and steam-explosion combined with concentrated sulfuric acid (0.5% concentration, at 150 °C for 6 min). Adapted from [33].

In another experiment, Wilkins et al. successfully demonstrated the production of ethanol with reduced costs on enzymes from $10.00 per gallon to $0.80 per gallon [5,21]. An integrated system of citrus juice manufacturing and food processing associated with microbial digestion unit for the waste biomass produced during food processing would be an advantage over the conventional standalone industrial set ups. This integrated system would be able to supplement the main production of processed food and juice drinks with limonene (an organic green solvent), ethanol and methane (biofuel and biogas). The latter can facilitate the reduction of energy costs involved in food processing itself. The citrus peel waste biomass is a sustainable and renewable resource for the production of HMF and dimethyl furan. The latter possesses 40% higher energy density compared with ethanol. Citrus peels also contain pectin, hemicellulose and cellulose. These are insoluble carbohydrates and constitute 60% of the total carbohydrates [83]. Synthesis of HMF requires decomposition or depolymerization of insoluble carbohydrates which is brought upon by treating the biomass with ionic liquid solvents and catalysts and some metal halides [84]. HMF can be produced from biomass through a series of chemical reactions: (a) hydrolysis of glucan to glucose, (b) isomerization of glucose to fructose, and (c) dehydration of fructose into HMF. A somewhat similar process yields furfural from xylose. Both of these compounds have been selected as top 10 biomass derived platform compounds by the U.S. Department of Energy on the basis of their estimated processing cost, technical complexity, as raw material and its market potential [85]. Given its importance as a platform chemical, the global HMF market for 2022 is expected to grow up to 145 million USD [86]. Reportedly, more than 80 chemicals have been derived directly or indirectly from HMF and furfural, among these a number of aromatic compounds which lists among the top 50 chemicals sold globally [87].

The chemical properties of the participating ionic liquids play a crucial role in the breakdown of C-O-C bonds present in α-, or β-glycosidic linkages of cellulose polymeric molecules and enhances the rate of formation of HMF. Ionic liquids have an edge over conventional chemicals in terms of low toxicity, and non-flammability. Furthermore, these are considered as environment-friendly solvents, e.g., 1-octyl-3-methyl imidazolium chloride

(OMIMCI). Research on ionic liquids for the production of biofuel in terms of synthesis of HMF is extensively explored nowadays. In a study conducted by Pourbafrani et al. suggests a possible reduction of green-house gas emission up to 134% by utilizing ethanol fuel from citrus wastes as a light duty vehicle fuel. Furthermore, methane from the citrus biomass can be used in place of natural gas for the production of electricity which in turn can reduce the green-house gas emission by 77%. The additional by-product, *d*-limonene can be used as a green organic solvent and replace acetone for chemical applications. The left over residual digestate from the reactor can be utilized to manufacture organic manure for crop production instead of using synthetic fertilizers. This is also considered to be an advantage in terms of reducing the emission of green-house gases which are produced during the manufacture of synthetic fertilizers from petroleum-based materials or other non-renewable resources [88–90].

2.4.3. Biodiesel

Biodiesel is a category of fuel obtained from renewable resources, such as, vegetable oils and animal fats. The fuel is non-alkyl esters of long chain fatty acids. These are non-toxic, biodegradable and release minimal or insignificant amounts of non-toxic gases besides CO_2, NO_x, etc., upon combustion [91]. Biodiesel can also be obtained from the peel oil extracted from citrus peel waste followed by trans-esterification, filtration and distillation. Apart from biodiesel, glycerol is also produced. The steps involved in the production of biodiesel from orange peels are shown in the Figure 4 (Flowchart constructed from the information given in the Reference [91]).

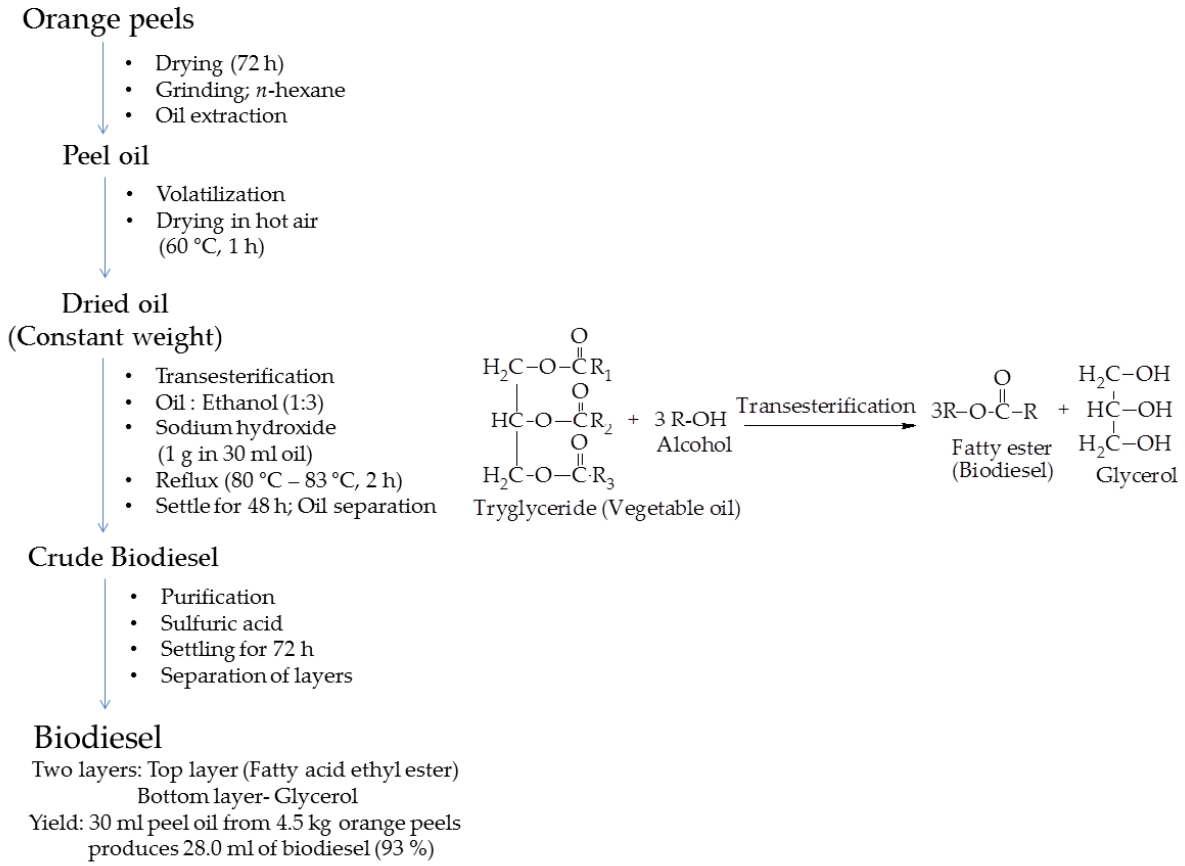


Figure 4. Steps involved in the process for the production of biodiesel. Adapted from [91].

2.5. Biorefinery

The National Renewable Energy Laboratory (NREL), in the year 2011, defined 'biorefinery' as a facility which combines biomass conversion process and equipment to produce biofuel (for energy and power), and important chemicals from biomass (food and agricultural waste). The fundamental steps required for a smooth operation of a biorefinery includes (a) pre-treatment of the biomass waste which may comprise standalone or a combination of both, viz., conventional methods (shredding, milling, filtration, centrifugation, solvent extraction, distillation, etc.) and modern methods (steam explosion, supercritical CO_2 extraction, etc.); (b) chemical treatment, in which usage of chemicals are included to control/adjust the pH of the biomass; (c) enzymatic hydrolysis, in which enzymes hydrolyses the complex polysaccharides into simpler monosaccharides, alcohol and organic acids; (d) conversion of monosaccharides and simple molecules and end products, viz., ethanol and lactic acid, products of chemical intermediates, e.g., levulinic acid, production of energy and power or utilization of the residue to produce farm manure or cattle feed; (e) treatment of waste water for reuse in domestic or agricultural purposes, solid waste valorization and control of air emissions, [10,92–94].

Soluble carbohydrates are glucose, fructose, and sucrose; and insoluble carbohydrates or cell-wall structures or the structural polysaccharides, viz., pectin, cellulose and hemicelluloses contain galacturonic acid, glucose, galactose, arabinose, and xyloses in their monomeric units. There has been observed an increase in ethanol production by 30% when the carbonate concentration is high compared to hydroxylate with low carbonate concentration in SSF tank. Ethanol production was also found to be enhanced by addition of yeast extract or ammonium sulfate supplements. Other nutrient supplements generally employed are magnesium sulfate, biotin, niacin, biotin-niacin mixture, potassium hydrogen sulfate, ammonium hydrogen sulfate; however, it did not show any huge difference in terms of benefits compared to no supplement or ammonium supplementation. An increase in the carbonate concentration have been found to decrease the inhibition of yeast by undissociated organic acids present in the hydroxylate which may range between 1.6 to 4.5 wt. % of total solids. These acids are mainly citric acid along with less to trace amounts of lactic, acetic, quinic and maleic acids which tend to lower the pH of the medium. Unlike supplements, high inoculum density has been observed to increase the ethanol yields. An inoculum loading of 126 $\pm$ 27 mg per 100 g wet citrus processing waste yielded 71% of ethanol compared with 45% yield from 12.6 mg/100 g of citrus processing waste at 37 °C in 24 h. the yield increased to 85% $\pm$ 1.75% at 48 h from 71% $\pm$ 3.01% at 24 h, at highest inoculum loading. *Kluyveromyces marxianus* is a thermotolerant strain and can carry out fermentation at higher temperature of 42 °C to 45 °C compared to *S. cerevisiae* which exhibits optimum activity at 37 °C. Enzymatic hydrolysis almost doubles the yield of total reducing sugars. Additionally, the amount of sugars fermentable by *S. cerevisiae* increases by 25–35%. On the other hand, potential ethanol yield from separate hydrolysis and fermentation process was observed to be 55–65 gallons per ton of dry peel solids [28,65,70]. The installation of biorefineries adjacent to the processing industries not only solves these negatively impacting problems but also enables to obtain valuable chemical compounds and sustainable production of fuels for energy demands. However, there exists a fundamental difference in handling citrus biomass which is pectin rich and other agricultural biomass which are lignin rich regarding processes to be carried out in a typical biorefinery. The process conditions for hydrolysis of citrus processing waste, kinetic parameters, design of the biomass reactor and overall engineering processes. The concept of biomass based biorefineries is observed as analogues to petroleum refineries which produce multiple products in addition to fuel. The fundamental steps carried out in a biorefinery have been summarized in Figure 5. The different fermentation processes and important chemicals that can be commercially synthesized from bio-oil/biofuel have been illustrated in Figures 6–9. The recent progress in this field has been summarized in Table 1.

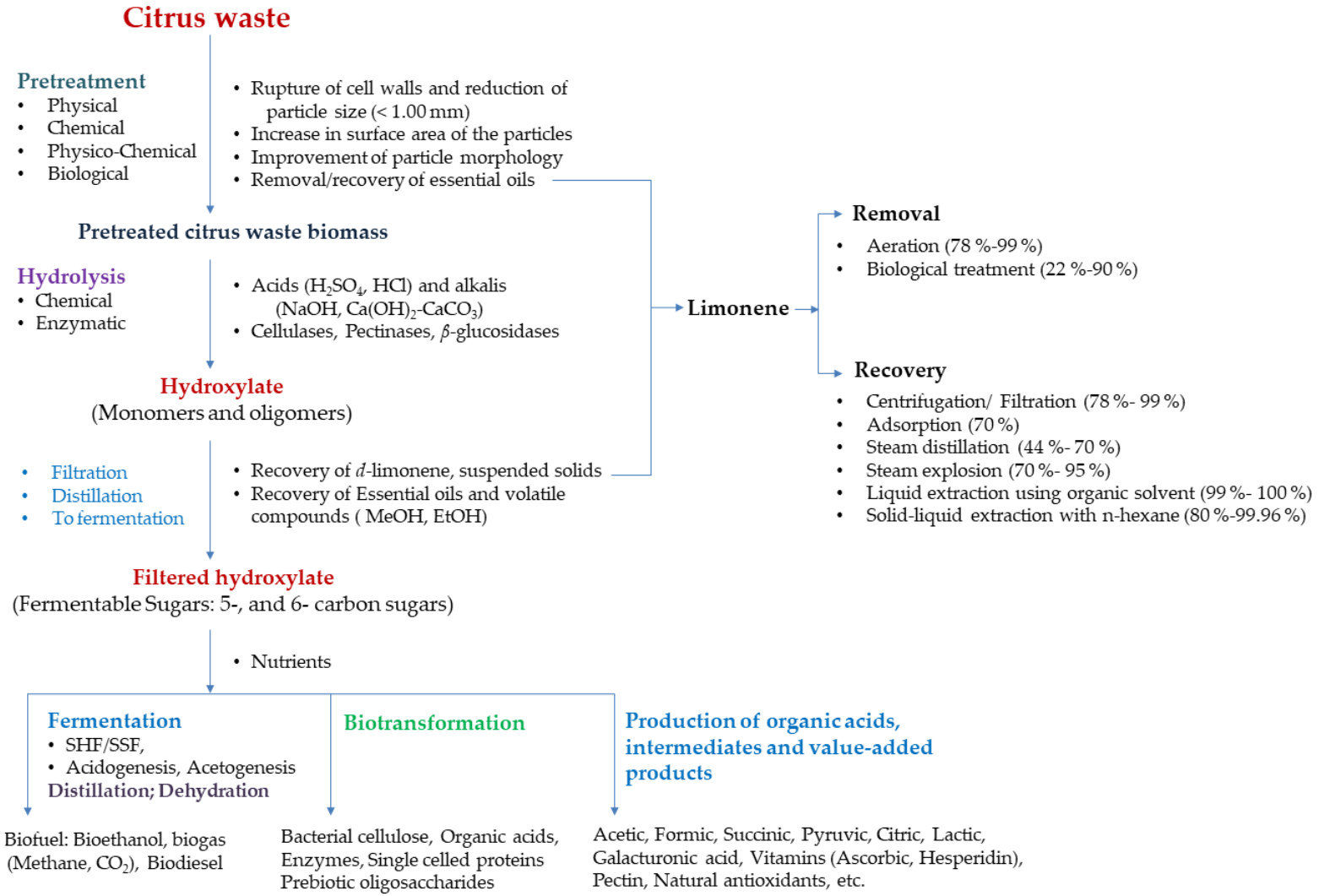


Figure 5. Fundamental processes associated with a typical biorefinery.

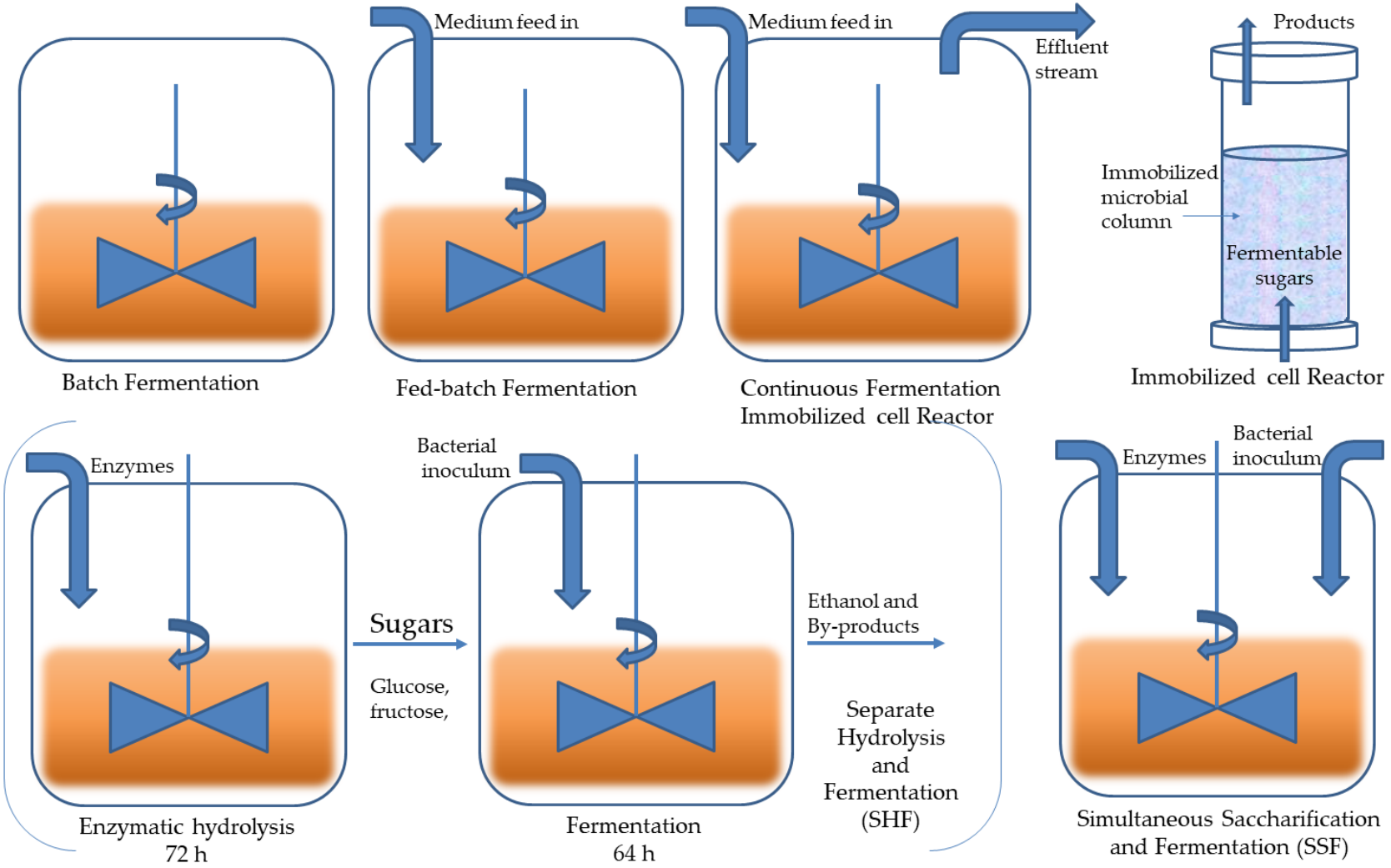


Figure 6. Methods of fermentation.

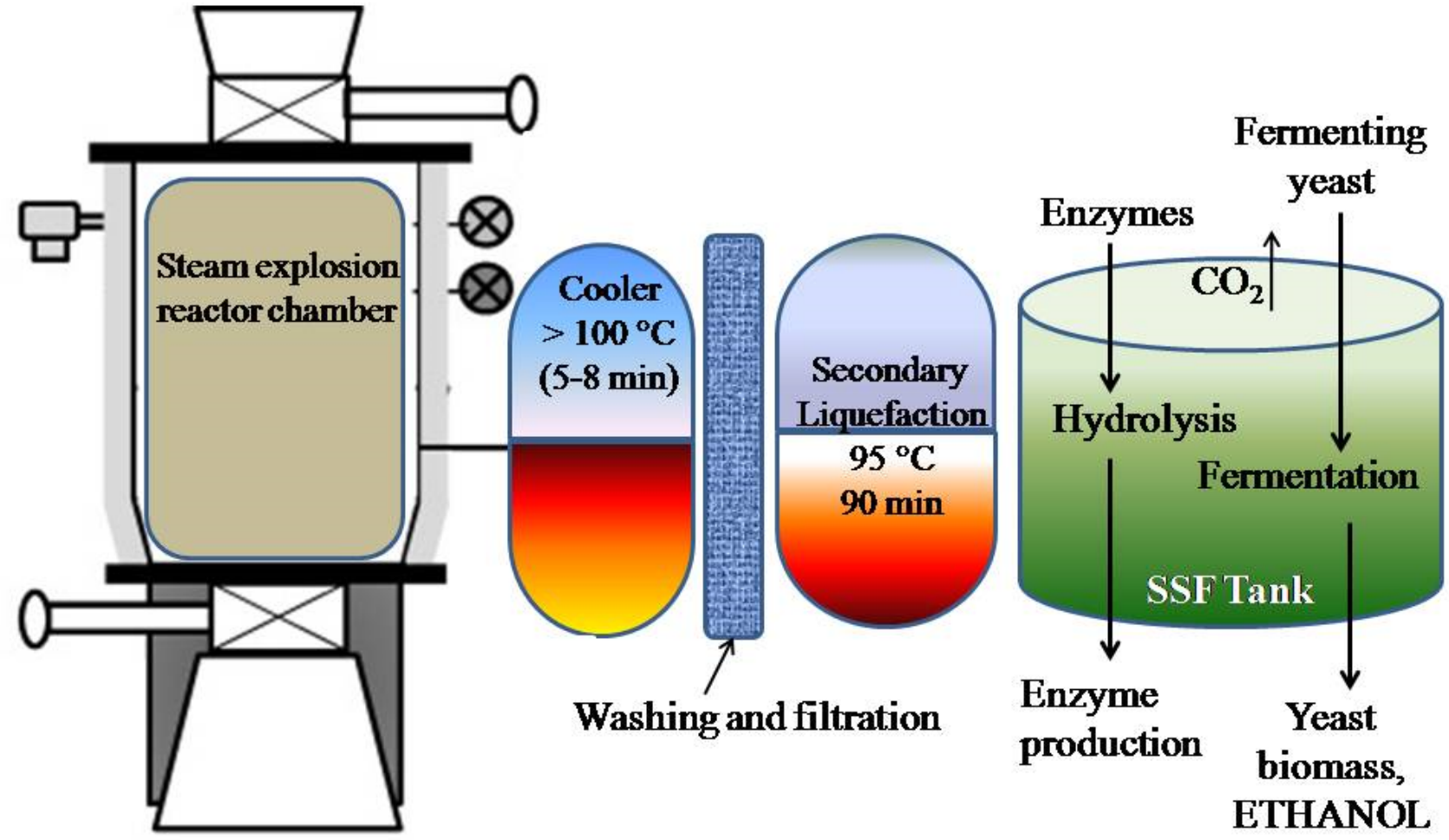


Figure 7. Simultaneous saccharification and fermentation assisted with steam explosion pre-treatment [4]. Reproduced with permission from Neelima et al., *Foods*, **2019**, pp. 8, 521–579.

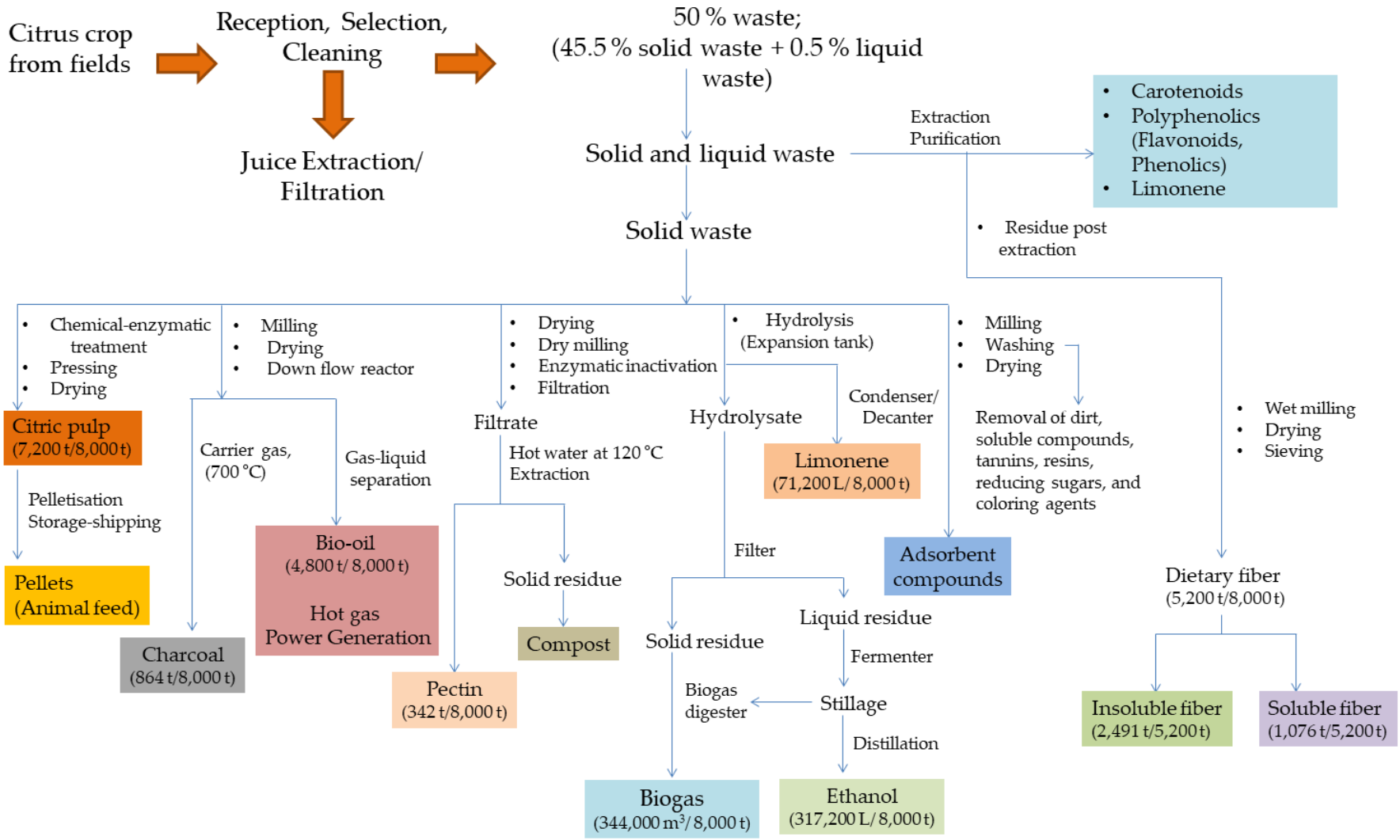


Figure 8. Citrus waste utilization for production of bioethanol, biofuel, biogas and various high value products.

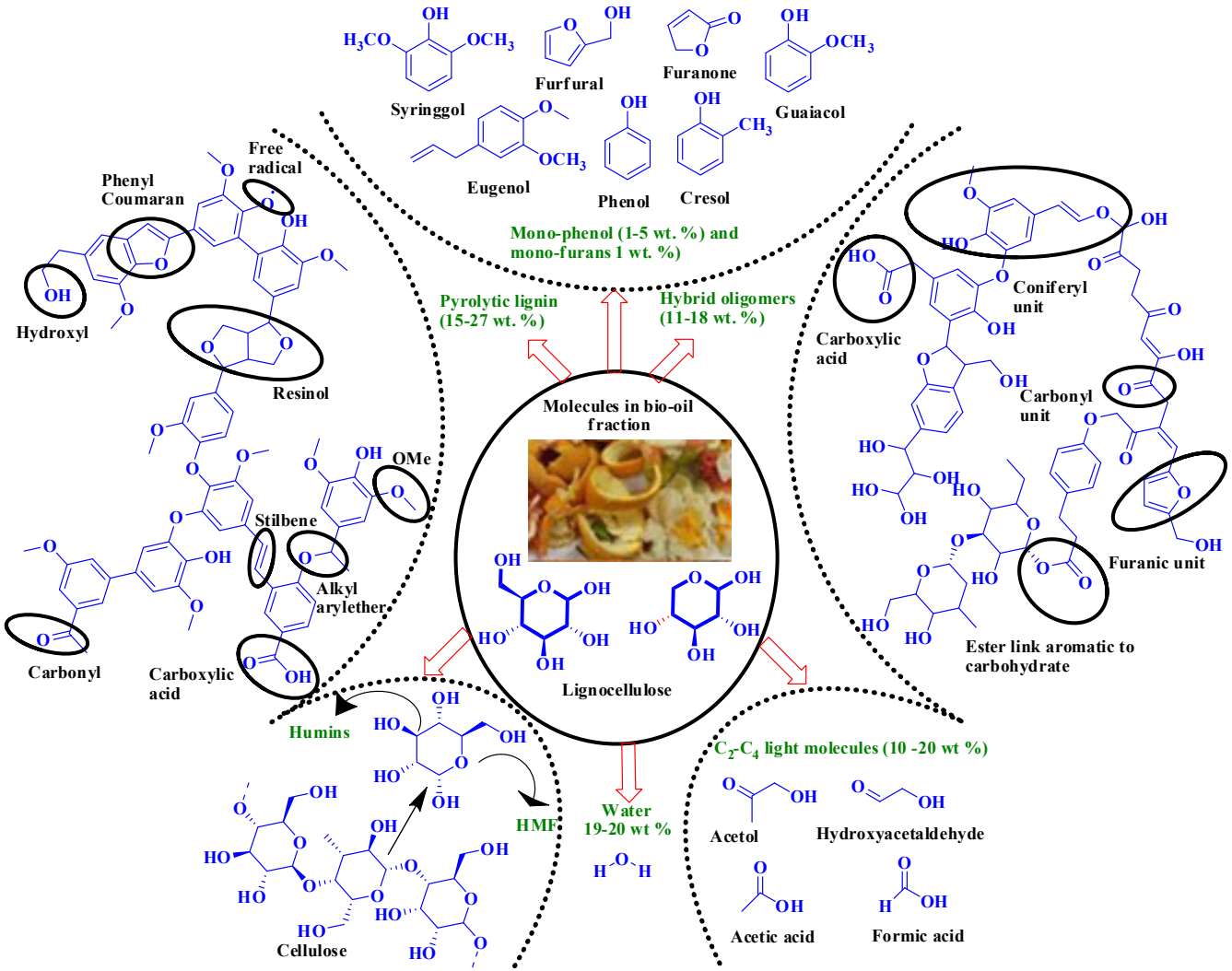


Figure 9. Chemical compounds potentially synthesized from bio-oil/biofuels derived from citrus waste biomass resource. (Adapted from [95–97]).

Table 1. Reports form recent research publications on the production of biofuels from citrus wastes.

Citrus Part	Procedure	Product and Yield	Application	Ref.
Citrus Peel and pulp	Peel and pulp is pulverized and pelletized; pyrolysis and gasification at 550–600 °C in molten sodium heat pipe furnace	Synthesis gas (H_2+CO), CO_2; biochar; H_2 yield ~51%	Hydrogen for clean energy	[98]
Citrus waste	Pyrolysis, gassification and catalytic reforming	H_2; biochar; H_2 yield 0.55 l/g of citrus pulp pellets	H_2 and clean carbon	[99]
Orange peels	Oil extraction in n-hexane using soxhelet; Drying at 60 °C, 1 h; Transesterification, 80–83 °C with ethanol, NaOH, 2 h; Settle for 48 h; Separation;	Biodiesel; fatty esters; 93% yield; in accordance with ASTM standards	Fuel in diesel engines	[91]
Mandarin peels (*Citrus unshiu*)	Grinding and lyophilisation at −50 °C; popping treatment, 150 °C, 10 min, 15 Kgf/cm pressure; enzymatic hydrolysis (cellulase, pectinase, xylanase, glucosidase); 50 mM sodium acetate, pH 4.8, 45 °C, 6 h; vacuum evaporation (condensation 1–10%); yeast fermentation, 30 °C, pH = 5 (*Saccharomyces cerevisae*) KCTC 7906; ssimultaneous saccharification and fermentation	Bioethanol; production: 46.2% (w/v) 90% yield; greater yield in shorter time (conventional methods take 12 h in enzymatic hydrolysis and 24–48 h during fermentation); low *d* -limonene; low cost	Fuel and industrial solvent	[34]
Mandarin peels	Peel waste is ground; sSteam explosion treatment—5 min, 160 °C, 6 Bar (removal of *d*-limonene); enzymatic hydrolysis, 24 h, 45 °C; yeast fermentation (*Saccharomyces cerevisae* CECT 1329), 37 °C, 4 days; ssimultaneous saccharification and fermentation	Bioethanol; production: 59.3% (w/v) *d*-limonene (90% recovery), galacturonic acid, citrus peel pulp; ethanol content of 50–60 L/1000 kg raw peel waste; steam explosion method reduces enzymatic dose requirement	Biofuel and industrial solvent/ chemical;	[66]
Orange peel (*Citrus sinensis*)	Dry at 40 °C; Steam treatment, 160 °C, 7 bar, 3–7 min; decompression at 50 mBar; oil extraction by thermochemical technique	*d*-limonene; 99% peel oil extraction	Industrial solvent for making soaps, perfumes, toiletries, etc.	[39]
Citrus waste	Ground and made slurry with H_2O; steam explosion, 150 °C, 20 min, 60 Bar; batch anaerobic digestion, 55 °C, microbe inoculum, 80% N_2 + 20% CO_2; shaking, 1–21 days	Methane; 107.4 m^3 methane and 8.4 L *d*-limonene per ton of citrus waste; steam explosion extracts *d*-limonene by 94.3%	Biogas;	[32]

Table 1. *Cont.*

Citrus Part	Procedure	Product and Yield	Application	Ref.
Citrus Waste	Thawing; grinding and slurry making in H_2O; digestion by methane producing bacteria in a membrane bioreactor, 55 °C, 30 days; organic biomass loading rate = 0.3–3.0 kg volatile solid/m^3/day	Methane; 73% methane production; methane production of 0.33 Nm^3/kg vs. compared with 0.05 Nm^3/kg vs. in traditional method	Biogas;	[100]
Orange peel	Dilute acid hydrolysis−0.5% w/v H_2SO_4, 150 °C; enzymatic hydrolysis; (cellulase, pectinase, *β*-glucosidase); fermentation (*Mucor indicus*)	Ethanol, glycerol; EtOH yield = 0.36 g per gram peel waste after 24 h; glycerol yield = 0.048 g/g in 24 h; chitosan recovery	Fuel and industrial solvent;	[101]
Citrus waste	Dilute acid hydrolysis; Explosive drainage, 150 °C, 6 min; Flashing; Anaerobic Digestion	Ethanol, pectin, *d*-limonene, biogas	Biofuel, biogas,	[22]
Orange peels (*Citrus sinensis*)	Biological treatment (*Penicillium digitatum*, *Penicillium italicum*); steam distillation, 100 °C, 1 atm., 60–180 min; ethanol extraction (70% ethanol), 60 min biochemical methane production post-treatments (1–3); 20–40 days, organic biomass loading rate 3 Kg volatile solid $m^{-3}d^{-1}$	Limonene, methane; yield per ton of citrus waste with 20% dry weight ethanol-39.64 L; methane-45 m^3; limonene- 8.9 L; pectin-38.8 Kg, 1. 22% 2. 44%; (BMP-36%, MPR-74%) 3. 100%; (BMP-34%, MPR-74%)	Biofuel and industrial chemical; limonene extraction;	[36]
Citrus Peel Waste	In-house enzyme production—*Aspergillus citrisporus* (KCCM11449); *Trichoderma longibrachiatum* (KCTC6507); limonene removal column (LRC) continuous immobilization yeast fermentation-immobilized reactor cell (ICR)—*Saccharomyces cerevisiae* KCTC7906	Ethanol, limonene; yield-93% LRC-ICR give 12 fold greater ethanol than ICR fermentation alone	Biofuel, industrial solvent/ chemical;	[41]
Orange Peel	Drying, 110 °C, 3d; pyrolysis, 1 Bar, inert atmosphere, 450 °C	Biochar cum biosorbent; Biosorption from aqueous solution in the following order→ Pb > Cu > Ni > Cd > Zn > Al; Biochar Calorific value- 10.9–19.3 MJ/Kg	Solid fuel; biosorbent;	[102]

Table 1. *Cont.*

Citrus Part	Procedure	Product and Yield	Application	Ref.
Orange peel (*Citrus sinensis* cv Valencia)	Milling; enzymes: pectinase (Pectinex Ultra SP), cellulase (Celluclast 1.5 L), glucosidase (Novozym 188); sodium acetate buffer with pH 4.8 hydrolysis and fermentation; microorganism (*Saccharomyces cerevisiae*	Enzymatic hydrolysis doubles the yield of total reducing sugars. Sugars fermentable by *S. cerevisiae* increases by 25–35%. Ethanol yield −4.7% w/v hydrolysis and fermentation produces 55–60 galons of ethanol per ton of dry peel waste compared with 40–50 galons of ethanol per ton of peels from fermentation alone	Ethanol	[28,41]
Orange peel	Milling; enzymes (pectinase, cellulase, glucosidase); hydrolysis and fermentation; microorganism (*Escherichia coli* KO11)	Fermentation by recombinant *E coli* KO11 increases the ethanol yield by 25–35% compared with fermentation by yeast; ethanol yield-2.76% *w/v*	Ethanol; small amounts of acetic acid and traces of lactic and formic acid	[28,41,70]
Orange peel	Steam explosion; enzymes: pectinase (Pectinex Ultra SP), cellulase (Celluclast 1.5 L), glucosidase (Novozym188); simultaneous saccharification and fermentation; microorganism (*S. cerevisiae*); yeast concentration of 0.7 g cells/100 g CPW; Temperature 37 °C; pH 6.0	Ethanol yield-3.96% w/v; ethanol production were reduced at pectinase loadings less than 25 IU/g peel dry matter, cellulase loadings less than 0.02 FPU/g peel dry matter, and beta-glucosidase loadings less than 13 IU/g peel dry matter; steam explosion removes 90% of *d*-limonene	Ethanol, *d*-limonene	[21,41]
Orange peel	Steam explosion (155 °C under 410 to 550 kPa, 2 min); enzymes (pectinase, cellulase, glucosidase); enzyme loading of 1 mL solution in water containing 60 IU pectinase, 0.035 FPU cellulase, and 0.81 IU beta-glucosidase g^{-1} dry CPW; simultaneous saccharification and fermentation; microorganism: (*Kluyveromyces marxianus*)-thermotolerant yeast (loading—1.26 ± 0.27 mg cells g-1 peel waste); temperature—40 °C; pH—4.8; 48 h	Ethanol yield-3.45% w/v (86%) high inoculum density increases the ethanol yield from 45% to 71% at 37 °C in 24 h and 86% in 48 h; *Kluyveromyces marxianus* is thermotolerant and can ferment at an elevated temperatures of 42 °C–45 °C	Ethanol, *d*-limonene	[41,72]

Table 1. *Cont.*

Citrus Part	Procedure	Product and Yield	Application	Ref.
Orange peel	Acidic steam explosion (160 °C for more than 4 min); acid treatment (pH 2.8); enzymes (1 mL per 100 g CPW): pectinase (Rapidase PNS), cellulase (Celluclast 1.5 L), glucosidase (Novozym 188); simultaneous saccharification and fermentation; microorganism (*S. cerevisiae*); addition of calcium carbonate to the SSF mixture (0.75 g for acid and untreated CPW, 0.3 g for CPW at initial 6.8 pH, no carbonate for CPW at initial 8.2 pH), 1 mL of antibiotics per 100 g CPW (5 mg/mL each of Neomycin and Streptomycin in water)	Ethanol-2.7% w/v (76% to 94%); ethanol yields were observed to be not affected after SSF using either acid or base adjustment to CPW prior to pretreatment at 160 °C for limonene removal	Ethanol, *d*-limonene	[41,71]
Lemon peel	Steam-explosion; enzymes (pectinase, cellulase, glucosidase); simultaneous saccharification and fermentation; microorganism (*S. cerevisiae*)	Ethanol- 67.8% w/v; ethanol production of 60 L/1000 kg fresh lemon peel biomass; the pre-treatment reduces the residual content of essential oils below 0.025% and significantly decreases the hydrolytic enzyme requirements	Ethanol, lemon EOS, *d*-limonene, galacturonic acid; citrus pulp pellets	[41,67]

3. Organic Acids

Citric, malic and ascorbic acids are main components of citric fruit juices. It also consists other acids in varying and trace amounts, namely, lactic, oxalic, formic, tartaric acid, pyruvic acid, benzoic, succinic, and so on. The citric and malic acids, and their salts in combination, act as a buffering system by resisting any substantial alteration of pH (acidity) of the juice upon dilution.

3.1. Citric Acid

Citric acid is a six-carbon tricarboxylic acid. In natural form, it can be extracted from citrus juices or pressed juice from pith and pulp residue obtained as a waste from the citrus food processing industries. The pith and pulp residues are treated with calcium oxide or lime which converts citric acid present in the substrate into calcium citrate and the latter is precipitated out. The precipitate is separated by filtration. This is then treated by sulfuric acid to recover the citric acid. In the current time, the annual global production of citric acid exceeds 1.4 million tons and the demand continues to increase every year. Citric acid can also be produced from fermentation led by *Aspergillus niger* and industrially by chemical synthesis. Chemical synthesis is relatively an expensive process. The microbial production of citric acid can be carried out by employing any of the following microbial strains, namely, *Penicillium janthinellum*, *Penicillium restrictum*, *Trichoderma viride*, *Mucor piriformis*, *Ustulina vulgaris*, and various species of genera, *Botrytis*, *Ascochyta*, *Absidia*, *Talaromyces*, *Acremonium* and *Eupenicillium* [103]. In a research carried out by Aravantinos-Zafiris et al. involved three different strains of *A. niger* and the results suggest that *A. niger* NRRL 599 produced best yield compared with *A. niger* NRRL 364 and NRRL 567, respectively [104]. Reports show that approximately, 90% of the world's total citric acid supply comes from fermentation process. The two most popular fermentation process, viz., solid state fermentation and submerged liquid surface fermentation processes are known to yield best results. Solid state fermentation has an edge over submerged fermentation technology as it requires less water quantities. The latter results in reduction of water activity, and consequently, improved aeration in the reactor. This facilitates exchanged gases, viz., oxygen and carbon dioxide between the gas phase and substrate medium [4,12].

In another investigation, Torrado et al. carried out a comparative study on production of citric acid using both the techniques, viz., solid state fermentation and submerged fermentation method. They employed *A. niger* CECT 2090 in solid state fermentation technique which yielded 193.2 mg of citric acid per gram of dry orange peel under 85 h of operation. The yield was greater than that obtained from submerged fermentation method. Furthermore, the solid-state fermentation method did not require any additional supplies of microbial growth nutrients. However, sterilization is a common requirement in both the methods. As citric acid does not undergo phase transition, therefore, it is possible to obtain purified citric acid in good amounts without involving complicated steps. To further simplify the manufacturing process and obtain enhanced quantities of highly purified citric acid product, specific techniques for extraction and purification are employed, namely, solvent extraction, spray technique, adsorption on a suitable substrate, ion exchange and membrane separation methods for purification. Post-production, the methods for estimation and determination of purity of citric acid and appropriate suitability in terms of food grade standards are employed [105,106]. The most commonly utilized solvents are n-octylalcoholtridodecylamine, isoalkane, mixture of butylacetate and N, N-disubstituted alkylamide [107,108]. The crystallized form of citric acid is obtained after washing and distribution of extra solvent. In addition, the impurities can also be removed by purging carbon dioxide into the concentrated solution of citric acid in acetone. This creates an anti-solution effect of carbon dioxide [109]. One of the advantages of solvent extraction process over chemical synthesis or extraction is that it allows prevention of usage of harsh chemicals such as lime and sulfuric acid [110]. The modern techniques, namely adsorption on a suitable substrate and ion exchange method allow rapid recovery along with high efficiency and specificity. However, these techniques require analytical

grade chemicals which involve an expensive budget. Other modern techniques include membrane separation which utilizes a thin polymeric membrane. The latter allows selective transport of solute and solvent molecules, thereby facilitating purification of citric acid. Some of the popular membrane separation processes are electrodialysis, reverse osmosis, nanofiltration, ultra-filtration, and so on. Electrodialysis was first introduced for the purification of citric acid in 1970s [106]. However, in the later years, relatively less expensive methods were invented to reduce the cost of production significantly. Citric acid is known for its anti-oxidant properties due to its chelating ability towards metal ions. The latter participate in catalytic oxidation. Chelation also blocks the nutrient molecules present in the food material which might be consumed by the microbes and its growth. This saves the food from rapid spoilage [111].

3.2. Lactic, Succinic, Pyruvic and Acetic Acids

Fermentation of citrus pressed juice obtained from citrus pith and pulp residue led by lactobacillus (*Lactobacillus delbrueckii*, ATCC 9649) produces lactic acid. A maximum of 90% efficiency of lactic acid production can be achieved in 4–5 days [112]. Post-fermentation, purification is carried out by addition of lime and precipitation of calcium lactate followed by filtration and crystallization. The crystallization of lactic acid is brought upon by dissolving the precipitate in acid and treating the same with activated carbon. This solution is filtered again and allowed to settle which eventually give rise to purified crystals of calcium lactate. This is then treated with sulfuric acid to precipitate calcium sulfate and lactic acid is left behind in the solution which is treated with ammonium hydroxide to convert lactic acid into ammonium lactate. During the entire fermentation process, the lactic acid remains in the medium as ammonium lactate and eventually converted into butyl lactate. The latter is separated by fractional distillation method by the virtue of its volatility. Post-separation, lactate is concentrated and further hydrolyzed to produce purified lactic acid [112].

Succinic acid is a dicarboxylic acid and conventionally manufactured via hydrogenation of maleic anhydride followed by hydration. Succinic acid is also produced from fermentation on citrus waste biomass brought upon by a range of microorganisms, e.g., *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens*, *Basfia succiniciproducens* and *Actinobaccillus succinogenes*. Reports suggest that *Actinobaccillus succinogenes* yield the best results in terms of utilization of carbon dioxide and transform into carboxylic acid, valorization of monosaccharides and amounts of succinic acid under anaerobic conditions [113]. Besides fermentation, succinic acid is also produced by means of chemical routes. Some of the popular chemical methods are catalytic hydrogenation, paraffin oxidation, electrolytic reduction of maleic acid or maleic anhydride. The succinic acid market is currently ~16,000 t/year. It is estimated that the price competitiveness of succinic acid can replace petroleum-derived maleic anhydride (~213,000 t/year) [14,59]. Li et al. reported on production of succinate from orange peel waste and wheat straw via consolidated bio-processing. The method combines the processes, viz., cellulose hydrolysis and sugar fermentation employing cellulolytic bacteria *Fibrobactor succinogenes* S85. The pretreated substrate biomass yielded 1.9 g/L of succinate from orange peels and 2.0 g/L from wheat straw, respectively [114]. Solid state cultures of *Geotrichum candidum* on acid pretreated orange peels waste biomass have been reported with high digestibility (73–88%) in vitro. Succinic acid finds wide application in the production of commercially important industrial products, such as polyester, polyols, polybutylene, succinate-terephthalate resins, pigments, coating materials, as a flavoring agent and sweetening agent in food and pharmaceutical industries [4,12,115,116].

Pyruvic acid is produced from citrus waste biomass by fermentation incorporating different strains of microbial yeasts, such as, *Debaryomyces coudertii* IFO 1381, *Candida utilis* IFO 0396, *Hansenula fabianii* IFO 1370, *Hansenula miso* IFO 0146 and *Debaryomyces nilssoni* IFO 1255. Reports on systematic practical fermentation using these microbes suggest that *Candida utilis* IFO 0396 and *Debaryomyces coudertii* IFO 1381 are capable of yielding the

maximum [117,118]. Pyruvic acid is utilized as a reagent in clinical analysis. Besides this, pyruvic acid is also used as a base material or substrate for enzymatic synthesis of amino acids, viz., tyrosine and tryptophan [113,118]. A comparison of yields of pyruvic acid by various strains of yeast has been displayed in Figure 10 (Adapted from [118]).

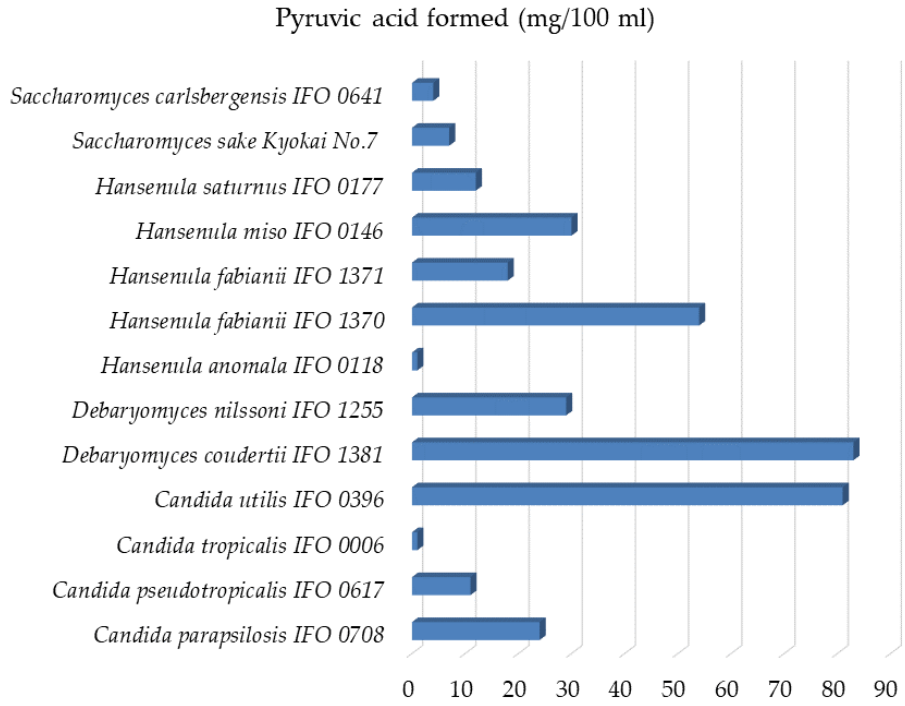


Figure 10. Productivity of pyruvic acid of various yeast strains cultured with shaking at 30 °C for 24 h with composition having peel extract as glucose or carbon source (5%), NH_4Cl (0.5%), K_2HPO_4 (0.1%), $MgSO_4 \cdot 7H_2O$ (0.01%) and yeast extract (0.01%) at a pH of 6.0. Adapted from [118].

Vinegar is 5–20% acetic acid by volume in aqueous medium. It is produced by fermentation of sugars present in citrus waste biomass obtained from food processing industries. The microbial yeast, *Saccharomyces cerevisiae* led fermentation on biomass produces ethanol which is further oxidized into acetic acid or vinegar by the action of *Acetobactor aceti*. The final product can be concentrated to increase the strength for commercial purposes. The vinegar produced from orange waste biomass reflects a fine fragrance and flavor and considered to be more in demands in food processing purposes and salad ingredients. Vinegar finds huge applications in presentation of food and utilized as a flavoring agent to salads, vegetables and sausages [4,12]. The various steps involved in the production of organic acids, viz., (a) succinic acid (b), pyruvic acid (c), citric acid (d) vinegar (e), lactic acid (f) ascorbic acid, and (g) source material for vitamin-B complex from citrus processing wastes by fermentation have been summarized in Figure 11.

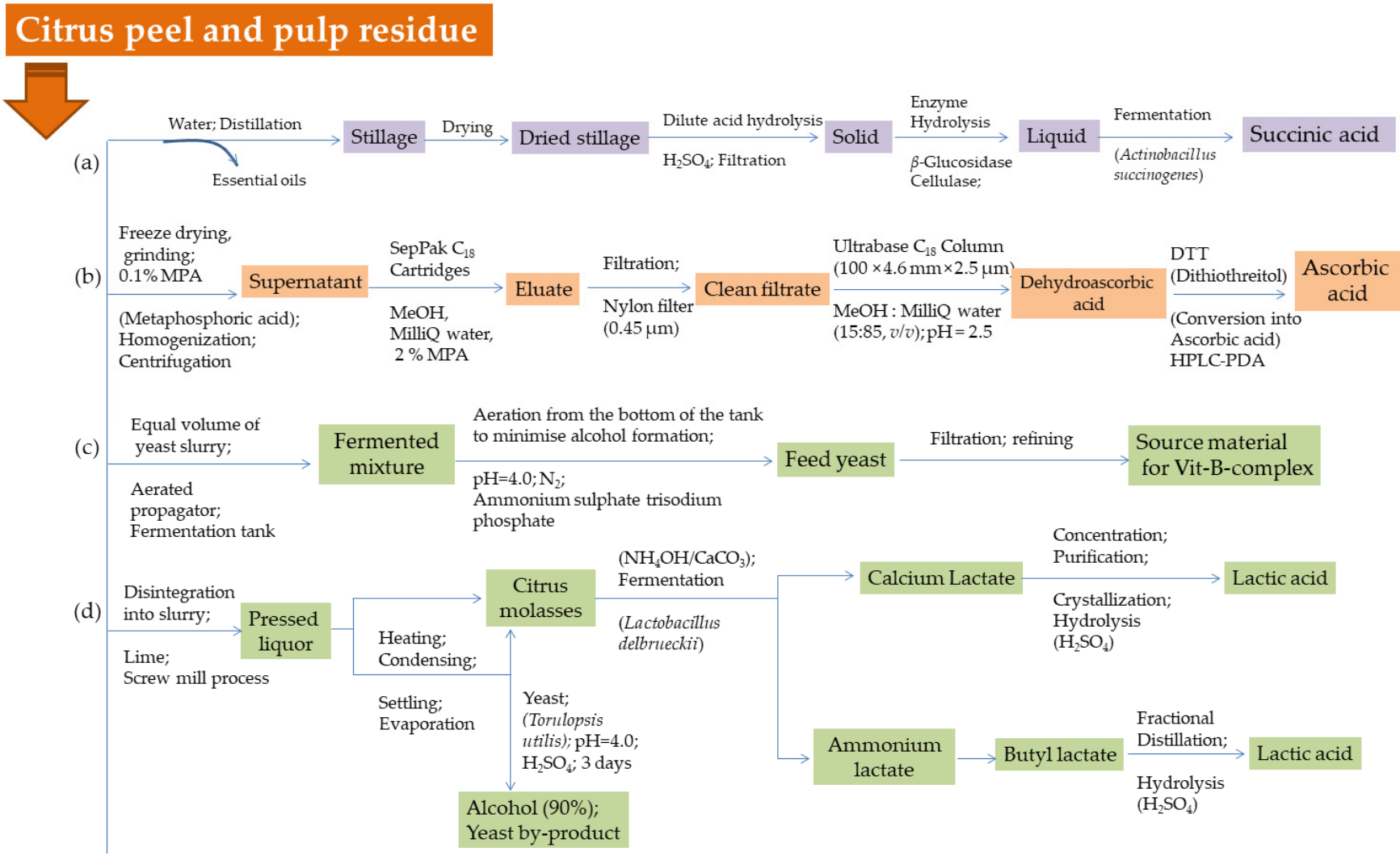


Figure 11. *Cont.*

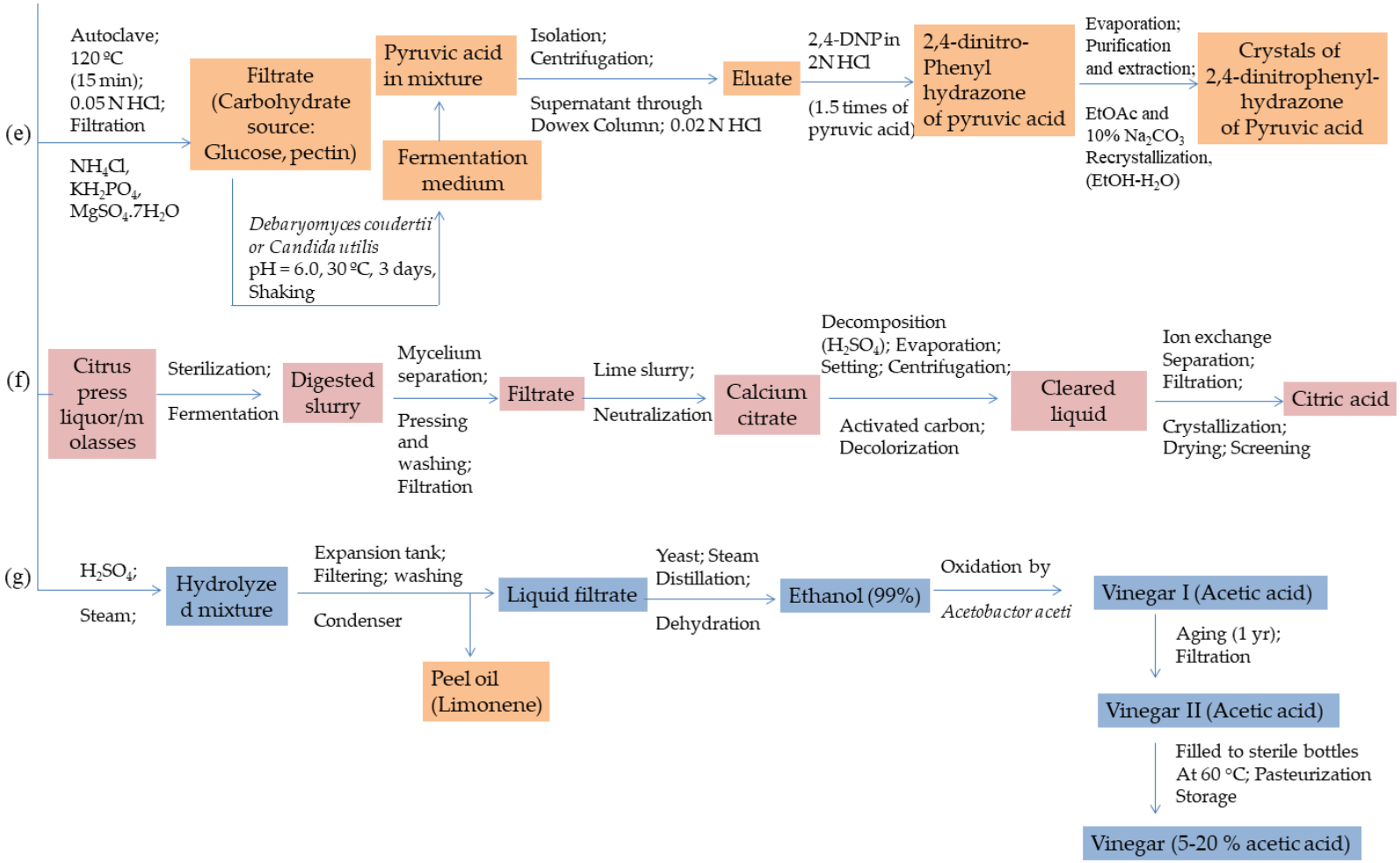


Figure 11. Steps involved in the production of organic acids, viz., (**a**) succinic acid (**b**), ascorbic acid (**c**), source material for vitamin-B complex (**d**) lactic acid (**e**) pyruvic acid, (**f**) citric acid, and (**g**) vinegar from processing of citrus waste.

4. Valuable Products

(a) *Vitamins*: Similar to many different carboxylic acids, viz., citric, pyruvic, succinic and acetic (in the form of vinegar), the vitamins, namely, vitamin-C (ascorbic acid + dehydroascorbic acid) in ample amounts and pro-vitamin A, B-complex, riboflavin and co-factors in trace amounts are also found in citrus fruit juices. Riboflavin is commercially produced from molasses employing yeast strains, viz., *Ashbya gossypi* and *Eremothecium ashbyii*. The microbes in the fermentation media require additional nutrient supplements, such as proteins and carbohydrate sources to produce good yields. A near neutral pH of 6.6 to 8.0 is generally required for optimal microbial action. Gaden et al. reported a maximum yield of 0.7 g of riboflavin per liter of diluted molasses obtained from citrus waste residue. The fermentation medium contained, *A. gossypii* was found to be incapable of transforming riboflavin from the fermentable ingredients present in citrus molasses [119]. Hesperidin, also known as Vitamin-P, is another high-valued by-product is recovered from the solid residue collected after an acidic pretreatment of peel waste in quantities ranging between 3.7% and 4.5% of dry mass [4,14,115].

(b) *Pectin*: Pectin has been reported to be composed of 17 different monosaccharides. Annual consumption of pectin worldwide exceeds 45 million kilograms with a global market value of 1.0 billion USD in 2019 and expected to achieve 1.5 billion USD by the year 2025. Pectin is conventionally extracted by using chemicals, such as, strong acids, e.g., oxalic acid, HCl. HNO_3 and H_2SO_4. In current times, there has been a shift from chemical to green methods regards extraction of pectin. Commercial enzymes, e.g., multienzyme complexes containing pectinolytic, cellulolytic, hemicellulolytic and proteolytic enzymes have been used to extract pectin from citrus waste biomass [120–123]. Besides enzymatic extraction, several thermal and mechanical techniques, viz., ultrasound assisted extraction, autoclave extraction, extrusion cooking or microwave assisted extraction and subcritical water extraction [4,14,92,124–129].

(c) *Single cell protein*: Single cell protein is a crude, refined and edible microbial protein obtained from biological substrates from agricultural and industrial processing wastes. Single cell proteins are extracted commercially from algae, fungi, yeast and bacteria which possesses very high quantities of proteins in their cell bodies. Bacterial single cell proteins contain 50–80% protein by dry weight. *Spirulina* and *Chlorella* are major sources of revenue generation in this market. Citrus waste biomass can be a relatively inexpensive feedstock to manufacture single cell proteins. Citrus processing waste is rich in cellulose, hemicellulose but have low quantities of lignin. The composition is ideal for producing feed for ruminants and microbial proteins or single cell proteins. The methods for obtaining single cell proteins are simultaneous saccharification and fermentation, solid state fermentation and separate hydrolysis and fermentation employing unicellular microorganisms. Fermentation of bergamot peels using strains of *Penicillium* sp. have been observed to improve nutritional value of the feed by increase in the amounts of crude proteins, crude fats and structural carbohydrates [130]. Solid-state fermentation of orange and lemon pulps by *P. roqueforti* Pr2 have been also observed to be an effective bioconversion in terms of enhancing quantities of proteins and lipid content in the yield [131]. Significant amounts of single cell proteins and high-activity crude pectinases have been produced from hydrolysis of lemon pulps by *A. niger* and *T. viride*. The highest protein level in the yield was observed after 14 days by using *A. niger*, whereas, *T. viride* yielded higher nitrogen content (31.9%) compared to *A. niger* which yielded (25.6%) [132].

(d) *Prebiotic oligosaccharides*: Prebiotics are selectively fermented ingredients which allow specific changes in the composition and/or activities of microbial flora in the gastrointestinal tract and helps in sustaining well-being and health. The best-known prebiotic oligosaccharides are fructooligosaccharides, galactooligosaccharides, lactulose, pectic oligosaccharides (POS), etc. POS helps in regulating lipid and glucose metabolism with reduced glycemic response and blood cholesterol levels. Besides

this, POS have also been observed to exhibit anti-cancer, anti-obesity, antibacterial, antioxidant and immunological properties. These also help in growth of friendly bacteria, such as bifidobacteria and *lactobacilli* in the gastrointestinal tract and limit the growth of pathogenic bacteria. Citrus peel albedo, and pectin obtained from citrus are considered as good source materials for obtaining prebiotics. The common methods are hydrolysis by enzymes, microwave and autoclave extraction, non-isothermal processing with hot compressed water (autohydrolysis or hydrothermal treatments of citrus peel substrates [133–137]. Olano-Martin et al. obtained POS from hydrolysis by pectin enzymes from citrus and apples in an enzyme membrane reactor [134]. Simultaneously, pectinolytic enzymes as well as pectinase and cellulase were produced from bergamot peels and orange peels, respectively, along with POS production. Furthermore, the solid residue obtained after the extraction of pectin from citrus waste is a good resource for the extraction of pectic oligosaccharides which is present in the soluble fraction [122].

(e) *Bacterial cellulose*: *It* is a linear homopolymer composed of *β*-1,4-linked D-glucopyranose. Citrus fruits are rich in soluble sugars, cellulose, hemicellulose and pectin and can be used as a sustainable and renewable feedstock for the production of bacterial cellulose (BC). One of the recently reported methods for obtaining bacterial cellulose utilized citrus peels citrus peels (lemon, mandarin, orange and grapefruit). The citrus peel waste biomass was hydrolyzed using dilute acid subjected for microbial biotransformation by *Komagataeibacter hansenii* GA2016 for 21 days at 28–32 °C under static conditions. The yield was found to be between 2.06 and 3.92% and the BCs produced from citrus peel hydrolysates were examined to be similar to the BC produced from commercial methods, possessed high water holding capacity, thin fiber diameter, high the thermal stability and high crystallinity [138]. Kuo et al. employed *Gluconacetobacter xylinus* for the production of bacterial cellulose. The nutrient medium consisted acetic acid buffer or nitrogen source and added to the orange peel media. G. Xylinus directly utilized soluble sugars and the production was found to be 4.2–6.32 times higher than that achieved with traditional Hestrin and Schramm (HS) medium [139]. The bacterial cellulose has been found to be free from other polysaccharide contamination, e.g., lignin or hemicellulose. It is an ultrafine 3D structural network of cellulose nanofibers (3–8 nm in diameter). It has specific high water holding capacity, great elasticity, significantly high wet strength and remarkable conformability. It can be recovered as a pure compound and find useful applications in biomaterials, viz., artificial skin, artificial cardiovascular tissues, scaffolds for tissue regeneration and wound coverage [14].

(f) *Enzymes*: Enzymes are biocatalysts and find immense applications in biotransformation of complex organic molecules into simple ones. Citrus peels have unique composition and a natural resource for extraction of a number of enzymes. Peroxidases are one of the most important biocatalysts, but its synthesis is low yielding as well as expensive. This limits its application. In recent years, many modern techniques have been introduced for enzyme bio-separation from natural resources. Three-phase partitioning (TPP) is one of the modest bio-separation techniques which bring satisfactory results. This method involves addition of a suitable salt, mostly ammonium sulfate to the aqueous suspension of crude extract obtained from citrus waste biomass followed by addition of tertiary butanol (Figure 12). Normally, tertiary butanol is completely miscible with aqueous phase, but it separates out at the top of the aqueous phase upon addition of ammonium sulfate in adequate concentration to the reaction medium. Besides peroxide extraction from orange peels (*Citrus sinensis*), the triple phase partitioning technique has also been used in extraction, separation, isolation and purification of a number of enzymes from natural resources, viz., invertase from tomato and yeasts; protease from papaya; cellulase, pectinase, xylanase from a number of plant resources, and so on [140–142]. The factors affecting the extraction process are duration of extraction, concentration of ammonium sulfate in the

reaction mixture, pH of the medium, ratio of substrate to t-butanol, temperature and speed of rotation organization of the reaction mixture [143]. This technique has been reported to obtain peroxidases with purity up to 93.96% from the orange peel waste biomass in the reaction mixture containing 50% of ammonium sulfate at a pH of 6 and temperature 30 °C. The mixture was supplied with tertiary butanol (feed to t-butyl ratio of 1:1.5 v/v) and centrifuged for 80 min. Besides pectinases and peroxidases, cellulases and hemicellulases are also extracted from citrus peels. These are included in multienzyme complexes. Cellulase enzymes comprise endo-1,4-β-D-glucanase, exo-1,4-β-glucanase and β-D-glucanase. These enzymes find applications in feed, fuel and chemical industries and employed in processing of lignocellulose materials. Hemicellulytic enzymes comprise β-1,4-endoxylanase, acetyl xylan esterase and phenolic acid (ferulic and *p*-coumaric acid) esterase. For extraction of these enzymes, both fermentation methods, viz., submerged and simultaneous saccharification and fermentation [14].

Enzymes find applications in efficient juice extraction, degumming of plant fibers, extraction of vegetable oil, bleaching pf paper, fermentation of tea and coffee, waste water treatment and removal of harmful substances of alcohol beverages and food processing [144]. Besides citrus waste biomass, enzymes are largely extracted from a range of natural resources, mainly agricultural wastes or residues, agricultural-based industrial wastes and wastes from food processing industries. Some of the notable examples are wheat bran, apple pomace, cranberry pomace, strawberry pomace, coffee and cocoa, citrus waste (lemon, orange, etc.), sugarcane bagasse, and so on [145–150]. Solid state fermentation is another commonly applied process for the production of enzymes. In this method, microorganisms, basically bacteria and fungi are cultivated and inoculated over moistened substrate in a solid-state fermenter [151]. Post fermentation, the ingredients are filtered off and the supernatant is separated. The enzymes are present in the supernatant. The enzymes are in crude form and harvested by means of centrifugation at 10,000–12,000$\times$ g rpm for 15 min at 4 °C. This is then treated with ammonium sulfate and the concentration can be enhanced up to 90% purity. The concentrated enzyme in the suspended in 0.01 M TrisHCl subjected to dialysis. The solution still contains partially purified enzymes. This solution is lyophilized and subjected to gel-filtration column chromatography using 0.01 mM TrisHCl buffer at a pH of 6.0 to obtain highly purified enzyme [152]. Some of the industrially important enzymes, namely pectinase, cellulases, xylanases and invertase (EC3.2.1.26) are reported to be extracted from microbial biodegradation and fermentation of orange peels by the action of certain fungal strains from genera *Aspergillus*, *Fusarium*, *Neurospora* and *Penicillium* [153]. Furthermore, pectinolytic, cellulolytic and xylanolytic enzymes can also be obtained from simple growth medium by employing the aforementioned microbial strains. The growth media contains citrus waste biomass and mineral supplements to promote healthy growth of microorganisms. One of the main advantages of solid-state fermentation is the formation and production of fermentable sugars as a by-product. The sugars are converted into bioethanol [14,31]. Triple phase partitioning method for obtaining enzymes from citrus processing waste is shown in Figure 12. The commonly employed microorganisms in the production of enzymes from citrus processing wastes have been summarized in Table 2.

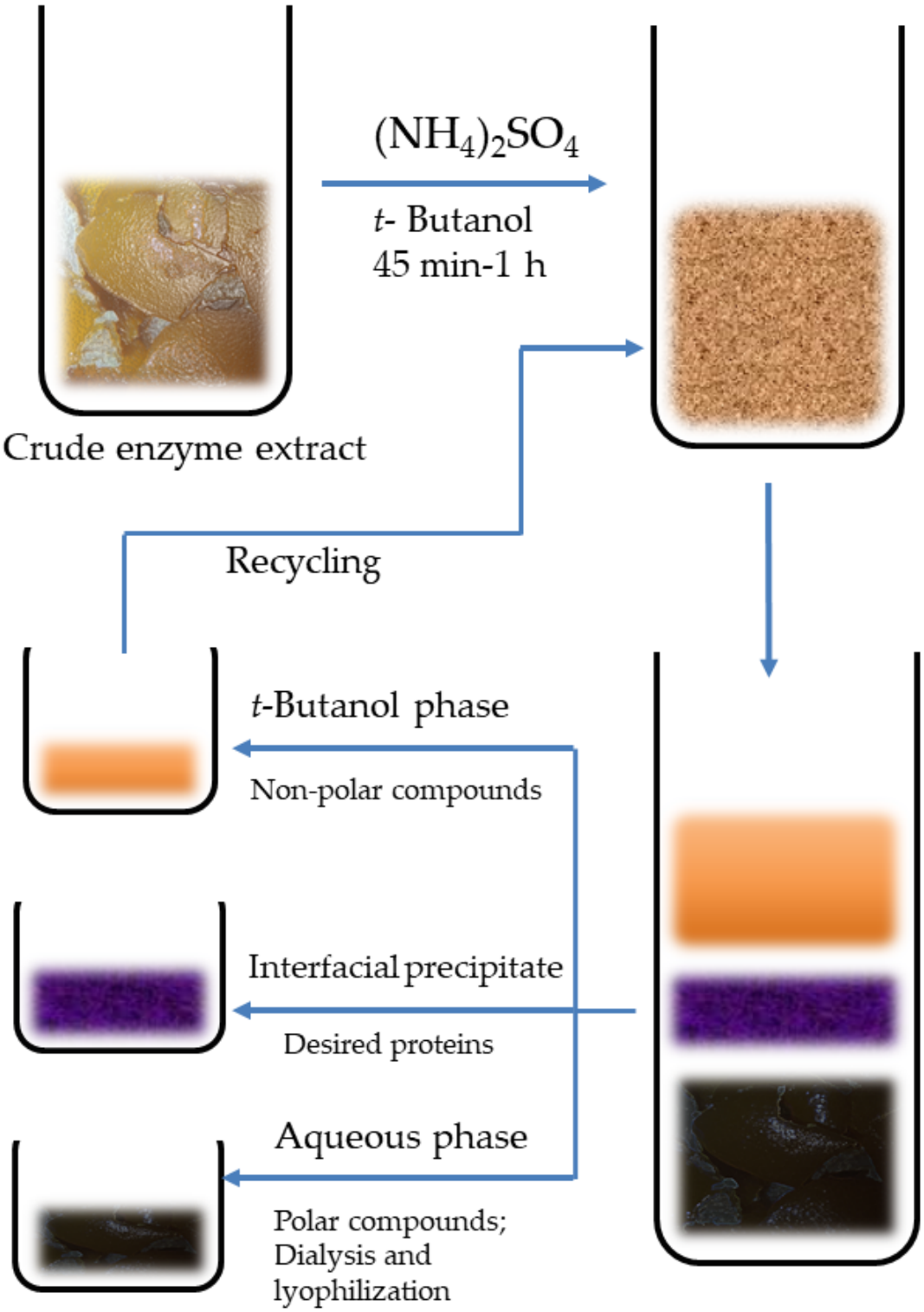


Figure 12. Triple phase partitioning method for obtaining enzymes from citrus processing waste. The enzymes are present in the interfacial precipitate and separated and purified to obtain enzyme.

Table 2. Microbial strains employed to produce enzymes from citrus waste.

Enzyme (Name and Type)	Function/Applications	Citrus Waste Part Utilized	Microbe Strain Applied; Reaction/Process Parameters	Ref.
Pectinase, Cellulase, Xylanase, Amylase and Lipase	In the extraction of the major components (starch and lipids) of plant materials	Orange (*Citrus sinensis* var. Balady) peels	*Aspergillus niger A-20;* pH 4.0–5.0; Temperature 45–50 °C	[152,154,155]
Polygalacturonase, Pectate, Lyase, Xylanase, Invertase	Degrade orange waste to various useful products	Dry citrus peels	*Aspergillus niger BTL*, pH 5.0; Temperature 49 ± 1 °C	[153]
Pectinase, Cellulase	Production of sugars, cellulose, hemicellulose and pectin from citrate waste	Orange peel and pulp	*Chaetomium* spp. *(KC-06)* pH 5.0–7.0; Temperature 25 °C	[155]
Hydrolytic (Pectinase), Oxidative (Laccase) enzymes	Bioconversion of lignocellulose	Orange (*Citrus sinensis* (L.) Osb.) pulp, including membrane tissueand peel	*Pleurotus pulmonarius* Temperature-28 °C in the dark	[156]
Endoglucanase, xylanase, invertase	Production of sugar	Dry orange peels	*Fusarium oxysporum F3* pH 6.0; Temperature 49 ± 1 °C	[153]
Endoglucanase	Degradation of cellulose	Dry orange peels	*Neurospora crassa DSM 1129* pH 5.0; Temperature 49 ± 1 °C	[153]
Endoglucanase, invertase	Production of sugar	Dry orange peels	*Penicillium decumbens* pH 5.0; Temperature 49 ± 1 °C	[153]
Polygalacturonase	Liquefaction and solubilization of uranic acid and decrease in pectin content	Orange finisher pulp	*Rhizopus oryzae* pH 5.0	[157]
Cellulase, Pectinase	Extraction of free sugars from citrus waste	Citrus waste	*Mucor indicus* pH 5.5; Temperature 30 °C	[158]
Cellulase, Pectinase	Extraction of free sugars from citrus waste	Citrus waste	*Rhizopus oryzae* pH 5.5; Temperature 35 °C	[158]
Polygalacturonase, Pectin lyase	Pectin degradation	Orange bagasse	*Aspergillus giganteus* pH 3.5; Temperature 30 °C	[159]
Cellulase	Bacterial cellulose production	Citrus peel and pomace	*Komagataeibacter xylinus* pH 3.5; Temperature 30 °C	[160]
Pectin lyase	Pectin degradation	Orange bagasse	*Aspergillus giganteus* pH 3.5; Temperature 30 °C	[159]

5. Economic Aspects

Production of bioethanol from the citrus processing wastes is accompanied by the simultaneous production of *d*-limonene, methane (biogas), bioactive molecules and pectin at commercial scale which add up to economic benefits. Apart of these, the capital generated from these by-products is utilized in procurement and cultivation of enzymes, acids and microbial strains required for hydrolysis, and to some extent paying for the utility bills involved in energy, electricity and apparatus/equipment maintenance. Improved technologies and continuous innovations have helped immensely to scale up the production of bioethanol and by-products efficiently. The commercial production is still in the demonstration phase. According to assumptions based on calculations, a plant with production capacity of ~152,000 m^3 of ethanol per year where the ethanol price is USD 475.51 per m^3 provides a net gain of revenues of USD 90.00 per m^3 without *d*-limonene. On the other hand the gain is USD 169.00 per m^3 when *d*-limonene was included as a co-product [5,22]. Furthermore, it is estimated that a citrus processing waste based biorefinery would require ~3.3 million tons of fresh waste every year to make the process economically profitable.

Research aspects to be focused on to scale up the production can be summed up in the following points: (a) pre-treatment of citrus processing waste and hydrolysis of the same; enzyme loading, enzymatic reaction rates, yields of fermentable sugars, ethanol and overall co-production of limonene, methane, pectin and biologically active compounds; (b) kinetics and thermodynamics of pre-treatment processes on citrus waste: degradation of waste as a function of temperature and time; (c) upgradation and modernization of pre-treatment process in terms of yielding appropriate particle size of the raw material, surface area and morphology for enhanced action of hydrolysis and efficient results; (d) development of suitable processes to recover and obtain the by-products and important chemicals, such as polyphenols, carotenoids, sugars pectin, *d*-limonene, methanol and galacturonic acid along with bio-ethanol; and (e) statistical records on critical process data for small-to-medium-to-large sized bio-refinery units linked to citrus/food processing plants with waste generation capacity of 36,000–360,000 tons per year [10]. The cost of raw materials or feed-stock is a crucial aspect in the production of biofuel from waste-biomass. Besides this, the cost involved in the maintenance of different apparatuses and instruments processing unit(s) in the bio-refinery or the reactor, chemicals, enzymes, microbial strains and culture facilities, utilities (steam, water, electricity, etc.), consumables, equipment installation, labor, overhead charges and taxes of the state. During production of ethanol from biomass, lignin is generally obtained as a residue and consumed to generate steam and electricity, thereby reducing the utility costs considerably. However, during the production of ethanol from citrus waste biomass lignin is not recovered as a residue, but recovery of limonene as a valuable solvent and a co-product is economically significant (Figure 13).

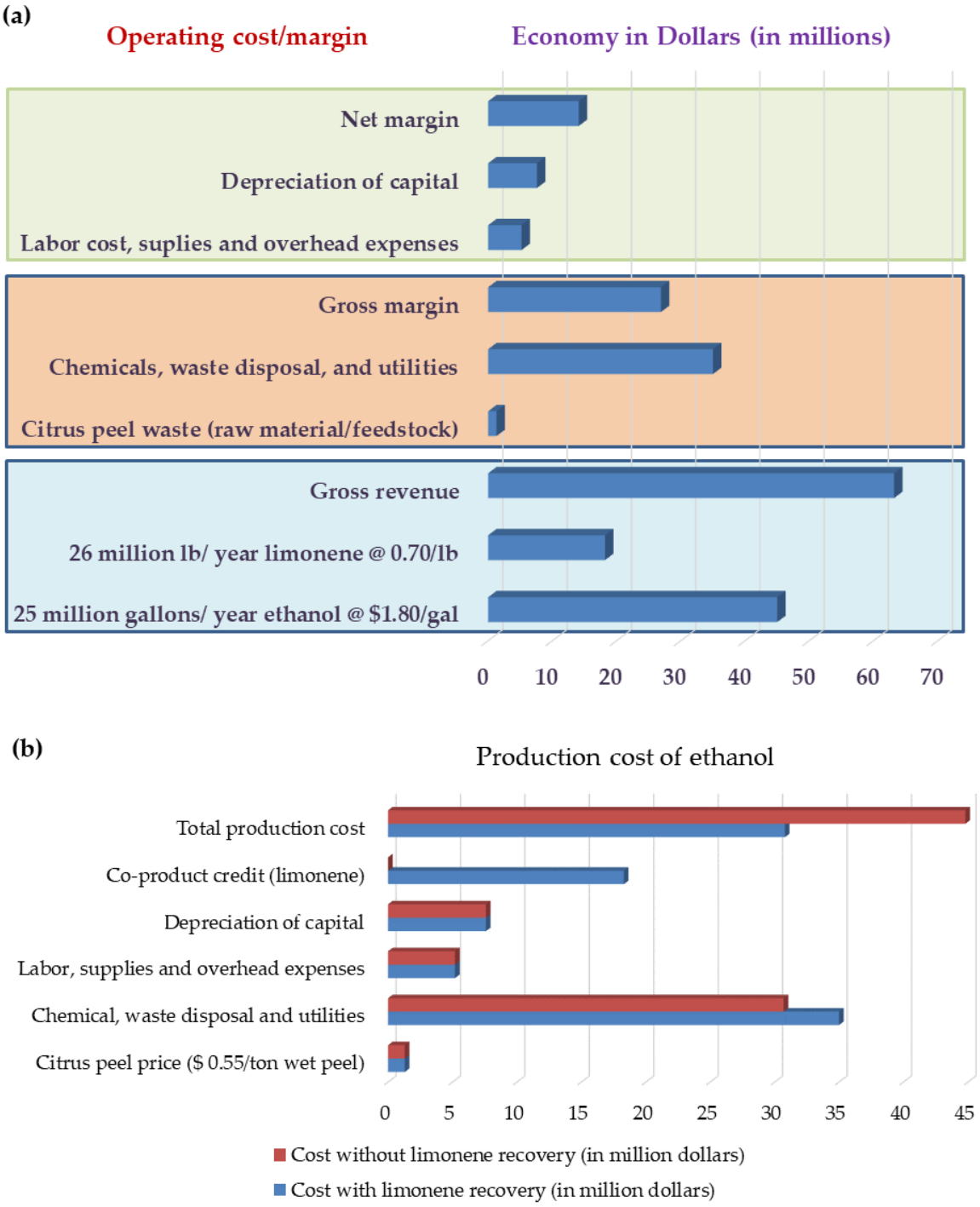


Figure 13. Citrus based ethanol economies; a comparative analysis of gross revenue: (**a**) production cost for the citrus peel waste-to-ethanol process with and without the recovery of limonene (capacity: 25 million gal/year) (**b**). Adapted from [5].

A bioethanol processing unit installed adjacent to huge citrus food processing industries across the world is established to produce 10 million gallons of ethanol per annum. This suggests that production of ethanol from citrus waste biomass is beneficial. However, installing a small unit would be more expensive and disadvantageous [5]. In the current time, it is yet to establish an installation of industrial scale ethanol production based on citrus waste biomass and limited to laboratory demonstration only. Patsalou et al. reported an efficient biorefinery strategy for the production of bioethanol and methane employing three different strains of yeast, viz., *Pichia kudriavzevii* KVMP10 and *Kluyveromyces marxianus*

and *Saccharomyces cerevisiae*. They proposed a zero-waste strategy by combining multiple extraction steps along with fermentation processes to produce ethanol and methane. This includes removal of limonene and essential oils in the first step using distillation of the hydroxylates post pretreatment. The remaining residue was dried and subjected to dilute acid hydrolysis and extraction of pectin. Simultaneously, ethanol was removed by distillation. The remaining residue was then subjected to fermentation by different yeast strains in separate tanks (for comparison) to produce ethanol. Post ethanol production, the residue was again treated with dilute hydrolysis and subjected to anaerobic digestion for the production of methane. *P. kudriavzevii* KVMP10 was observed to produce the highest yield of 30.7 g/L from the fermentation process conducted at 42 °C followed by 18.6 g/L by *K. marxianus* and 8.6 g/L by *S. cerevisiae*. The methane production was 342 mLgvs-1 (volatile solid) [161].

Limonene is a green chemical which has minimal or no adverse effects to health or environment. It is generally recognized as safe in applications by Food and Drug Administration (FDA). It has aromatic properties and used as a stabilizing agent in non-alcoholic beverages, fruit juices, ice-creams, cakes and bakery items. Besides, it has therapeutic properties and used in traditional medicine and skin care products due to the presence of nutritive compounds in its composition. In the recent years, limonene is also finding applications in end-user industries, such as automobile, aerospace, wood and marine industries and the global demand is on continuous rise. Pectin finds applications as gelling agent, and a variety of food products, such as jam, jellies, yogurts and deserts. It reduces cooking time, improves texture of the processed food and enhances its shelf life. It is also used in wound healing preparations, medical adhesives and other pharmaceutical products. Pectin have been observed to rise in demands in the European and Asia-Pacific countries, particularly in the food and beverage industries based on processed and packaged food. Galacturonic acid is obtained from oxidation of galactose, a sugar, and a main component of pectin. In pectin it exists in polymeric form, i.e., polygalacturonic acid. It is in high demands for its application in chemical industries, as laboratory reagent, and personal care products. The global organic acid market is expanding due to the rising usage of organic acids in food, beverages, cosmetics, chemical and pharmaceutical industries, animal feed, as a substitute of antibiotic growth promoters (AGP) due to its antioxidant properties, preservation, acid regulation and flavor enhancement. Furthermore, extraction of a number of bioactive compounds, such as flavonoids, pectin, galacturonic acid, carotenoids, etc., which possesses commercial value in terms of their applications in food and pharmaceutical industries, renders the production of ethanol from citrus waste biomass more beneficial in terms of economic advantages [4,12,86,162–166].

The summary of different processes carried out in a typical biorefinery dealing with citrus wastes to produce biofuel, biogas and important chemicals of potential commercial value are presented in Figure 14. The recent market sizes of the products that can be systematically generated from a functional biorefinery are summarized in Table 3.

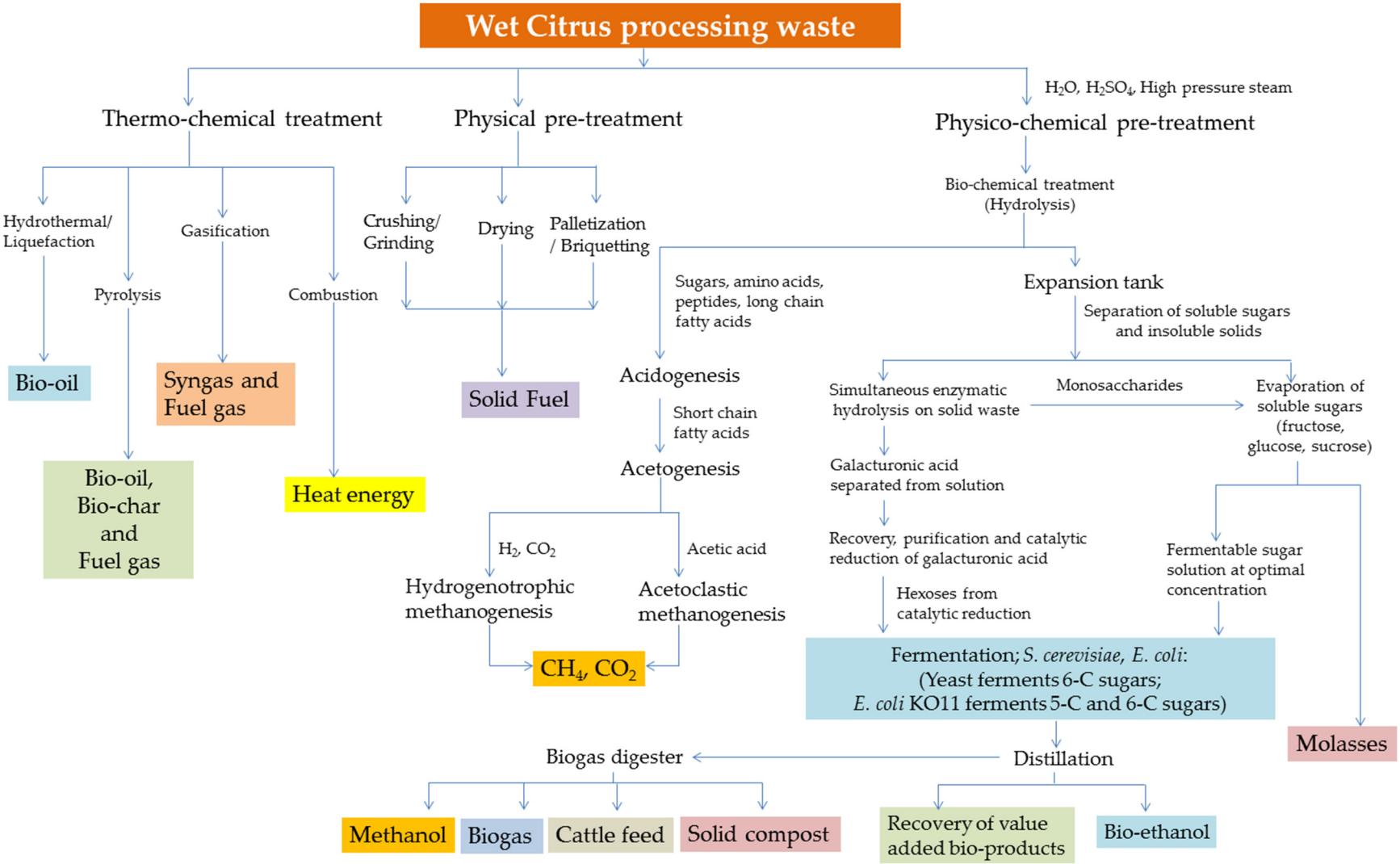


Figure 14. The treatment of citrus processing waste for the production of bioethanol and biofuel at a typical zero-waste biorefinery.

Table 3. Important chemicals of potential commercial value and their application.

By-Products	Application	Global Market Size *	Ref.
Limonene	Solvent, domestic household products, feedstock for new chemicals, cosmetics, pharmaceutical, food and beverage, personal care	323.2 million USD in 2020 379.2 million USD in 2026	[162]
Enzyme	Extraction of fruit juices, degumming of plant fibers, waste water treatment, vegetable oil extraction, fermentation of tea and coffee, bleaching of paper,	7.1 billion USD in 2017 17.2 billion USD by 2027	[167,168]
Citric acid	Medicinal citrates, confectionary, soft drinks, effervescent salts, silvering and engravings, dying and calico printing	2.5 billion USD in 2016 3.6 billion USD by 2020	[169]
Succinic acid	polymers, polyesters, polyols, polybutylene, surfactants, solvents, detergents, flavors, fragrances, succinate-terephthalate resins, pharmaceutics	181.6 million USD in 2019 237.8 million USD in 2022	[165]
Lactic acid	Flavoring agent, pH regulator, and preservative, cosmetic and food processing	997.2 million USD in 2020 1156.5 million USD by 2026	[163]
Single cell protein	Nutritional supplements, animal feed, food and beverage, pharmaceutical and biotechnology, cosmetic, and agriculture	5.3 billion USD in 2017 8.7 billion USD by 2023.	[170]
Prebiotic oligosaccharides	Healthy drinks, snack bars, bakery and confectionery, dietary supplements	4.0 billion USD in 2017 7.2 billion USD by 2023	[166]
Pectin	Food and beverages (gelling agents, thickener, stabilizer, fat replacer, jams, jellies, dairy products, beverages, bakery and confectionery)	1.0 billion USD in 2019 1.5 billion USD by 2025	[164]
5-hydroxy-methylfurfural (5-HMF)	Building block for new molecules for packaging, construction, textile, cosmetics, formaldehyde replacement in resins	145 million USD in 2022 120 million USD in 2017	[86]

* Market size representative of total production from all kinds of resources and not particularly from citrus waste feedstock. Production from citrus waste resources is believed to increase the contribution from natural resources.

6. Summary

Biological transformation involving microbes have been effective, energy efficient and environment friendly approach to produce a number of useful products from natural resources since ancient times. In modern times, these techniques can also be employed to solve our energy issues and fulfil the demands as well as dealing with difficult problems such as pollution from food processing wastes from industrial scale production units. In the present article, this aspect has been elaborately explained. In the future to come, energy and fuel demands are going to rise further. Development of technologies to harvest energy and desired fuel from renewable resources, such as waste biomass helps dealing with both the problems, viz., energy and waste management and pollution, effectively. Furthermore, increasing costs of gasoline or fossil fuel-based energy and problems related to pollution create ample space and motivation for the development of newer technologies. Citrus is the world's largest fruit crop cultivated in most of the tropical countries and global urbanization encourages modernization of food processing technologies which are based on industrial scale food processing, packaging and supplies. As a side effect, it creates huge amounts of wastes which can be utilized to produce ethanol, biogas, biofuel, organic acids, vitamins and enzymes by means of biotransformation. The development of potentially efficient strains of relevant microorganisms can enhance the yield or production of the desired chemicals. These products can be obtained at a lower cost or investment which will facilitate the lowering of production costs of many industrially important chemicals, such as lactic acid, succinic acid, acetic acid, etc., via biotransformation involving microbes.

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