Chapter 9
Production of Bioethanol from Food Industry Waste: Microbiology, Biochemistry and Technology

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9.1 Introduction

Ethanol, a solvent, extractant, and antifreeze, is used for synthesis of many solvents in the preparation of dyes, pharmaceuticals, lubricants, adhesives, detergents, pesticides, explosives, and resins for the manufacture of synthetic fibers and liquid fuel [163]. Ethanol is a major solvent in industries and ranks second only to water [152].

It is also employed as a solvent for resins, cosmetics and household cleaning products. The ethanol obtained from biomass-based waste materials or renewable sources is called as bioethanol and can be used as a fuel, chemical feedstock, and a solvent in various industries. Besides ethanol, biofuels containing butanol, propanol, 2-methyl 1-butanol, isobutanol, isopropanol, etc. are also employed. Bioethanol produced by fermentation is rapidly gaining popularity all over the world. The US, Brazil, Japan, France, U.K., Italy, Belgium, and The Netherlands are among the few countries widely using bioethanol for various uses [98]. It has certain advantages as petroleum substitutes, viz., alcohol can be produced from a number of renewable resources, alcohol as fuel burns cleaner than petroleum which is environmentally more acceptable. It is biodegradable and thus, checks pollution. It is far less toxic than fossil fuels. It can easily be integrated to the

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existing transport fuel system, i.e., up to 5% bioethanol can be blended with conventional fuel without the need for modification.

Gasohol (mixture of gasoline and alcohol) is widely used to run vehicles in developed countries. The use of alcohol as fuels is gaining vast popularity day-by-day and gasohol program is encouraged throughout the world. By encouraging bioethanol use, the rural economy could also receive a boost by growing the necessary crops. New technologies are being developed that are economically and strategically superior.

The interest in bioethanol as a fuel in response to petroleum price increase is the most significant factor influencing the world ethanol market. Recent oil shortage and escalating oil prices have led scientists to develop alternative energy sources to substitute petroleum. Global warming alerts and threats are on the rise due to the over utilization of fossil fuels. Alternative fuel sources like bioethanol and biodiesel are being produced to combat these threats. Bioethanol production from plant biomass has received considerable attention recently in order to mitigate global warming and demands for petroleum not from a finite resource and is a greenhouse gas emission. The road transport network accounts for 22% of all greenhouse gas emissions, and through the use of bioethanol as some of these emissions will be reduced as the fuel crops absorb CO_2. Also, blending bioethanol with petrol will help extend the life of diminishing oil supplies and ensure greater fuel security, avoiding heavy dependence on oil producing nations.

Biofuel obtained from renewable sources can be classified on the basis of their production techniques as given below:

- First-generation fuels refer to biofuels made from plants rich in oil and sugar.
- Second-generation biofuels (Biomass to liquid) are made from organic materials, such as straw, wood residues, agricultural residues, reclaimed wood, sawdust, and low value timber.
- Biofuels of third generation are produced from algae by using modern gene and nanotechnologies.
- Fourth-generation biofuels are produced from vegetable oil by using hydrolytic conversion/deoxygenating.

Tables 9.1 and 9.2 show biofuels of the four generations, their substrates and technological processes of their production.

It is apparent that different types of substrates can be employed to produce bioethanol. Accordingly, modification in its production technology has been made. The replacement of ethanol by ethylene is reversed in less industrialized nations. In Brazil and India, ethylene and its chemical derivates are produced by catalytic dehydration of fermentative ethanol [5].

The USA and Brazil are currently the primary producers of fuel ethanol, producing 49.6 and 38.3% of the 2007 global production, respectively. US bioethanol production is almost entirely from maize (corn) starch, which is converted into fermentable glucose by the addition of amylase and glucoamylase enzymes. In 2007, 24.6 billion L of ethanol was produced in the USA, that comprised of only 3.2% of gasoline consumption on an energy-equivalent basis [188].

Table 9.1 First- and second-generation biofuels, their feedstock, and technological processes of their production

Type of biofuel	Name	Biomass feedstock	Production process
First-generation (conventional) biofuel			
Bioethanol	Conventional bioethanol	Sugar beet, sugarcane, sugar, sorghum	Hydrolysis and fermentation
Pure plant oil	Pure plant oil (PPO)	Oil plants (e.g. rapeseed)	Cold-pressing/ extraction
Biodiesel fuel (plant energy)	Rape methyl-/ethyl ester (RME/REE) Fatty acids Methyl/ethyl ester (FAME/FAEE)	Oil plants (e.g. rape/ turnip rape seed, sunflower seeds, soy beans, etc.	Cold-pressing/ extraction/ transeste-rification
Biodiesel fuel	Fatty acids (waste grease)methyl/ethyl ester (FAME/FAEE)	Biodiesel cooking and deep fry grease	Transesterification
Biogas	Upgrade biogas	(Wet) biomass	Anaerobic digestion
BIO-ETBE		Bioethanol	Chemical synthesis
Second-generation Biofuel			
Bioethanol	Cellulose ethanol	Lignocelluloses	Up-gradation hydrolysis and fermentation
Synthetic biofuels	Mixed higher alcohols Bio-dimethyl ether	Lignocelluloses	Gasification and syntheses
Biodiesel (hybrid biodiesel from the first and second generation)	NExBTL	Plant oils and animal fats	Hydrogenaton (refining/ enrichment)
Biogas	SNG(synthetic natural gas)	Lignocelluloses	Gasification and syntheses
Bio-hydrogen		Lignocelluloses	Gasification and syntheses or biological process

Source [172]

Table 9.2 Third- and fourth-generation biofuels, their feedstock, and technological processes

Type of biofuel	Name	Biomass feedstock	Production process
Third-generation biofuels			
Biodiesel	*Oligae* Algae diesel	Algae	Gene and nanotechnology and esterification
Fourth-generation biofuels			
Bio gasoline Bio jet fuel Biodiesel	Synthetic oil	Vegetable oil (CENTIA^{TM} oil from algae)	Hydrolytic conversion/ deoxygenating

Source [37]

Table 9.3 Production cost of various chemicals using ethanol as feedstock

Chemical	Production cost ($/l)	
	From petroleum feedsock	From ethanol (at 40 g/l)
Acetaldehyde	60	66
Acetic acid	50	63
Butadiene	64	145
Ethylene	44	95
2-Ethyl alcohol	61	166

Source [139]

The production costs of various chemicals from ethanol and petroleum feedstocks are compared in Table 9.3. Clearly, the production of bioethanol from first generation is economically unreasonable because of discarding cellulose and hemicellulose which constitute the majority of carbon resources of plants. Furthermore, the biofuels of this generation also compete with food products intended for human consumption. Thus, second-generation bioethanol production is important as it allows improved CO_2 balance and make use of cheap, waste source which does not compete with human food products.

In brief, the use of ethanol as a biofuel is gaining increasing popularity. Although it is produced from several sources but the technologies using the waste material for its production is most attractive as it does not interfere with food particular substrates needed for the ever increasing world population. Different types of waste materials, their composition, biochemistry, microbiology, and the technology involved in bioethanol production have been reviewed in this chapter. New strategies and future thrust has also been briefly highlighted.

9.2 Raw Materials

9.2.1 Wheat Straw

Wheat (*Triticum aestivum* L.) is the world's most widely grown crop, cultivated in over 115 countries under a wide range of environmental conditions. Over the past 100 years, the yields of wheat have been increased and the annual global production of dry wheat in 2008 was estimated to be over 650 Tg [10]. Assuming residue/crop ratio of 1.3, about 850 Tg of wheat residues are annually produced which include straw as the major waste. The straw produced is left on the field, plowed back into the soil, burnt, or even removed from the land depending on the convenience of the landowner. Disposal of wheat straw by burning is viewed as a serious problem due to the increased concern over the health hazards of smoke generated [93]. Burning of wheat straw also results in production of large amounts of air pollutants including particulate matter, CO, and NO_2 [110]. Thus, finding an

Table 9.4 Composition of arable crop residues based on dry mass (DM) and potential for bioethanol production

Biomass	Residue/ crop ratio	DM (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Carbohydrates (%)	Ethanol (l/kg DM)
Barley	1.2	81.0	–	–	9.0	70.0	0.31
Maize (stover)	1	78.5	45	35	15–19	58.3	0.29
Oat	1.3	90.1	–	–	13.7	59.1	0.26
Rice straw	1.4	88.0	32–47	19–27	5.571	49.3	0.28
Sorghum	1.3	88.0	–	–	15.0	61.0	0.27
Wheat straw	1.3	90.1	33–40	20–25	16–20	54.0	0.29
Sugarcane Bagasse	0.6[a]	171	40–45	30–35	20–30	67.1	0.28

Source [95, 140, 144]
[a] kg of bagasse/kg of dry sugarcane

alternative way for disposal of surplus wheat straw is of paramount interest and an immediate necessity.

Wheat straw like any other biomass of lignocellulosic nature is a complex mixture of cellulose, hemicellulose, and lignin as three main components (Table 9.4), and a small amount of soluble substrates (also known as extractives) and ash. The overall chemical composition of wheat straws could slightly differ depending on the wheat species, soil, and climate conditions. The cellulose strands are bundled together and tightly packed in such a way that neither water nor enzyme can penetrate through the structure [104, 179]. Hemicellulose serves as a connection between lignin and cellulose fibers, and it is readily hydrolyzed by dilute acid or base, as well as hemicellulase enzyme. Lignin is covalently linked to cellulose and xylan (predominant hemicellulose carbohydrate polymer in wheat straw) such that lignin–cellulose–xylan interactions exert a great influence on the digestibility of lignocellulosic materials [104]. Due to this structural complexity of the lignocellulosic matrix, ethanol production from wheat straw requires at least four major unit operations including pretreatment, hydrolysis, fermentation, and distillation. Unlike sucrose or starch, lignocellulosic biomass such as wheat straw need to be pretreated to make cellulose accessible for efficient enzymatic depolymerization.

9.2.2 Sugarcane Bagasse

Sugarcane bagasse is the wastes from the sugar factory. It is obtained as a left-over material after the juice is extracted from the sugarcane. Sugarcane bagasse (SCB) was analyzed for its composition, structure, and surface properties (Table 9.4).

Because of its lower ash content, 1.9% [111], bagasse offers numerous advantages compared to other agro-based residues such as paddy straw, 16% [4], rice straw, 14.5% [60], and wheat straw, 9.2% [210]. In another study, SCB was obtained from a small sugarcane juice factory and milled for analysis of different types of fibers. It is important to note that most developments in SCB transformation into sugars and ethanol have a common scientific base with other lignocellulosic materials, due to considerable similarity in composition and structure.

9.2.3 Rice Straw

Rice straw, a waste from paddy processing, has several characteristics that make it a potential feedstock for fuel ethanol production. It has high cellulose and hemicellulose content that can be readily hydrolyzed into fermentable sugars. The chemical composition of feedstock has a major influence on the efficiency of bioenergy generation. The low feedstock quality of rice straw is primarily determined by a high ash content (10–17%) compared to wheat straw (around 3%) and also high silica content in ash (SiO_2 is 75% in rice and 55% in wheat) [205]. On the other hand, rice straw as feedstock has the advantage of having a relatively low total alkali content (Na_2O and K_2O typically comprise <15% of total ash), whereas wheat straw can typically have >25% alkali content in ash [12].

In terms of chemical composition, the straw predominantly contains cellulose (32–47%), hemicellulose (19–27%), and lignin (5–24%) [48, 116, 159, 204] as shown in Table 9.4. The pentoses are dominant in hemicellulose, in which xylose is the most important sugar (14.8–20.2%) [149].

9.2.4 Fruit and Vegetable Waste

9.2.4.1 Apple Pomace

Apple pomace is the solid phase resulting from pressing apples for juice, containing the pulp, peels, and cores. It accounts for 25–35% of the dry weight of processed apple. It has very high moisture content and can be easily decomposed by microorganisms. It is of yellow-to-brown color [7]. It is a rich source of many nutrients including carbohydrates, minerals, fibers except protein [162]. Apple pomace has high contents of carbohydrates with about 9.5–22.0% of fermentable sugar [174] which makes it a good substrate for fermentation while its low protein content indicates its unsuitability as animal feed [67, 86].

The amount of initial sugar content, however, depends upon the variety of apple processed, the processing conditions used, and the amount of filter aids added [66].

Table 9.5 Proximate composition of apple pomace

Constituents	Composition	
	Wet weight basis	Dry weight basis
Moisture (%)	66.4–78.2	3.97–5.40
Acidity (% malic acid)	NA	2.54–3.28
Total soluble solids (TSS ^{o}B)	NA	57.85
Total carbohydrate (%)	9.50–22.00	48.00–62.00
Glucose (%)	6.10	22.70
Fructose (%)	13.60	23.60
Sucrose (%)	NA	1.80
Xylose (%)	NA	0.06
pH	3.05–3.80	3.90
Vitamin-C (mg/100 g)	–	8.53–18.50
Soluble proteins (%)	NA	3.29
Protein (%)	1.03–1.82	4.45–5.67
Crude fiber (%)	4.30–10.50	4.70–48.72
Fat (ether extract, %)	0.82–1.43	3.49–3.90
Pectin (%)	1.50–2.50	3.50–14.32
Ash (%)	NA	1.60
Polyphenols (%)	NA	0.99
Amino acids (%)	NA	1.52
Minerals		
Potassium (%)	NA	0.95
Calcium (%)	NA	0.06
Sodium (%)	NA	0.20
Magnesium (%)	NA	0.02
Copper (mg/l)	NA	1.10
Zinc (mg/l)	NA	15.00
Manganese (mg/l)	NA	8.50–9.00
Iron (mg/l)	NA	230.00
Calorific value (kcal/100 g)	NA	295.00

Source [65, 84, 86, 92, 174]

Alcohol-soluble compounds (monosaccharides, oligosaccharides, and malic acid) accounted for 32–45 wt% of oven-dry pomace. Glucose and fructose are the major components of this fraction. Apple pomace is an acidic substrate and has considerable buffering capacity due to the presence of malic acid in it. Apple pomace has high levels of Biochemical Oxygen Demand/Chemical Oxygen Demand (BOD/COD) and is highly biodegradable. The proximate composition of apple pomace is shown in Table 9.5.

Table 9.6 Characteristics of banana peel and pineapple wastes

	Banana peel	Pineapple waste
Total solids[a]	10.68	7.80
Volatile solids[b]	86.65	89.40
Ash	13.35	10.60
Organic carbon	41.37	51.85
Total carbohydrates	23.44	35.00
Cellulose	11.11	19.80
Hemicellulose	5.36	11.70
Total soluble	35.89	30.00
Total nitrogen	1.06	0.95
C/N ratio	39:1	55:1

Source [132]
[a] Percent total weight
[b] Percent total solid unless otherwise mentioned

9.2.4.2 Banana Waste

Banana waste mainly comprises the peels and stalks. The physicochemical characteristics given in Table 9.6 clearly show that it can be used for ethanol production.

9.2.4.3 Pineapple Waste

Pineapple waste comprises the skin, seeds, and remaining parts after juice extraction. Cooked Sago is added to mill juice to enrich it with sugar to a level of 8% (w/w). The physicochemical characteristics of pineapple waste are given in Table 9.6 that shows its potential for ethanol production due to high carbohydrates/total solids content [183].

9.2.4.4 Orange Peel Waste

Orange waste is another substrate used for ethanol production. The proximate composition of orange waste and orange filtrate is given in Table 9.7.

9.2.4.5 Potato Peel Waste

The proximate composition of potato waste and potato filtrate (Table 9.7). Apparently, indicates its suitability for the production of ethanol.

Table 9.7 Proximate composition of orange and potato waste materials

Component (%Y)[a]	Orange waste (peel, pulp, and seeds)	Potato waste (peel and trimmings)	Orange filtrate	Potato filtrate
Dry matter	20.98	17.82	4.29	1.69
Alcohol-insoluble solids	63.00	62.70	19.60	22.49
Total soluble sugars	15.00	1.40	16.9	3.92
Reducing sugars	9.16	0.91	10.24	3.04
Pectin	20.93	3.39	2.62	0.41
Cellulose	10.59	2.20	2.19	0.14
Starch	<1.00	66.78	<1.00	44.81
Crude protein	6.53	14.70	0.53	3.31
Ash	3.78	7.65	0.78	0.82
Volatile solids	96.22	92.32	99.22	99.18
pH	4.30	5.99	4.30	5.99

Source [115]

[a] All components are expressed as percent dry weights except the dry matter that is the per cent wet weight. Values are expressed as the mean of three determinations (variation <5%)

9.2.5 Coffee Waste

Use of coffee waste as a substrate for ethanol fermentation has also been reported earlier [16].

9.2.6 Cheese Whey

Large amounts of whey produced is posing a serious problem all over the world for its proper utilization. Only a few countries have succeeded in utilizing their total whey production [147]. Whey is rich in lactose, a dimer of glucose and galactose unit, and can be fermented only by a selected number of yeasts. Because glucose and galactose are readily fermentable sugars, it is suggested that β-galactosidase treated whey could make a better substrate for industrial fermentation than untreated whey.

9.2.7 Spent Sulfite Liquor

The sulfite process involving the delignification of wood with acid bisulfite is widely used by mills in Europe and North America. While the lignin part is solubilized by combining with HSO_3, the wood cellulose largely remains undegraded. The hemicelluloses are hydrolyzed into monosaccharides. Spent sulfite liquor (SSL), which consists of lignin sulfonates, hexoses and pentoses,

polysaccharides, galacturonic and acetic acid, some resins and unconsumed bisulfite, and ash, creates a major pollution problem when discharged into receiving water. Being the source of different types of carbohydrates, it has the potential for conversion into ethanol.

9.2.8 Bioethanol from Algae

The production of motor fuel from algae has been subjected to research for decades. Now, there is an opportunity to produce bioethanol simultaneous to the third-generation biofuel—algae diesel (*Oligae*). Carbohydrates in algae oil can still be converted into starch that can be used for ethanol production after hydrolysis into simple sugars.

9.3 Microorganisms for Bioethanol Production

9.3.1 Microorganisms and Their Characteristics

Microorganisms are a key component of the technology used in different fermentation regimes, including ethanol. Diverse groups of microorganisms are capable of producing ethanol. These include yeasts, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, bacteria *Zymomonas mobilis*, fungus *Fusarium oxysporum*, yeast-like fungus *Pachysolen tannophylus*, and thermophilic bacteria [28]. *Saccharomyces cerevisae* and *Schizosaccharomyces pombe* represent the organism of choice for the industrial production of ethanol due to the following features:

- Capable of fermenting a diverse range of sugars and sole production of ethanol, CO_2 under anaerobic conditions
- Due to their comparatively bigger size, they flocculate well to supply clean wash to the still and the wash and distillate lack offensive odor
- Contamination problem is under control as the fermentation process operates at low pH and high sugar concentration
- Are genetically stable and ferment 20–25% (w/v) sugar in molasses solution completely

9.3.2 Substrate and Microorganisms

The substrates mainly used in alcoholic fermentation are sugars with ethanol as the main product. The ability is widely distributed among the microorganisms.

The species of *Saccharomyces* are the main alcohol producers amongst the yeast *Z. mobilis* can also produce ethanol from glucose, which otherwise only utilize hexoses [150]. Alcohol is not a predominant end product in other bacteria. Certain yeasts including *S. cerevisiae* can also ferment pentose sugar, xylose to ethanol though the yield is lower compared to the fermentation of hexoses. For industrial alcohol production, yeast strains are generally chosen from *S. cerevisiae*, *Saccharomyces ellipsoideus*, *Saccharomyces carlsbergensis*, *Saccharomyces fragilis*, and *S. pombe*. For whey fermentation, *Torula cremoris* or *Candida pseudotropicalis* is used. Yeasts are carefully selected for high growth and fermentation rate, high ethanol yield, ethanol and glucose tolerance, osmo-tolerance, low pH fermentation optimum, high temperature fermentation and general hardiness under physical and chemical stress. Ethanol and glucose tolerance allows the conversion of concentrated feeds into concentrated products reducing the energy requirements for distillation and stillage handling. The osmo-tolerance property allows the handling of relatively concentrated raw materials such as blackstrap molasses with its high salt content. The osmo-tolerance capacity it also allows the recycle of large protein of stillage liquids, thus reducing stillage handling costs. Low pH fermentation combats contamination by competing organisms by preventing their growth. High temperature tolerance simplifies fermenter cooling. General hardiness allows yeast to survive both the ordinary stress of handling as well as the stresses arising from plant upset. The years of careful selection by industrial use have led to the selection of yeast strains with these desirable characteristics. Many of the best strains of yeast are proprietary but others are available from the culture collections [33].

9.3.3 Lignocellulosic Material for Ethanolic Fermentation

Fermentation of the sugars generated from enzymatic hydrolysis of biomass is another important step where a lot of technical advances are needed to make lignocellulosic ethanol technology feasible. What is desired in an ideal organism for biomass-ethanol technology would be a high yield of ethanol, broad substrate utilization range, resistance to inhibitory compounds generated during the course of lignocellulose hydrolysis and ethanol fermentation, ability to withstand high sugar and alcohol concentrations, higher temperatures and lower pH, and minimal by-product formation [143]. Unfortunately, all these features seldom exist together in any wild organism and the need of the industry would be to develop an organism which will at least partially satisfy these requirements [208].

The ability to use the hemicellulose component in biomass feedstock is critical for any bioethanol project. *S. cerevisiae* and *Z. mobilis*, the commonly employed organisms used in alcohol fermentation, lack the ability to ferment hemicellulose and derived pentose (C5) sugars. While there are organisms that can ferment C5 sugars (e.g., *Pichia stipitis*, *Pachysolen tannophilus*, *Candida shehatae*), the efficiencies are low. These organisms also need microaerophilic conditions and are

sensitive to inhibitors, higher concentrations of ethanol, and lower pH [26]. Worldwide, a lot of R&D efforts are being directed to engineer organisms for fermenting both hexose (C6) and pentose (C5 sugars) with considerable amount of success [4]. There are a large number of microorganisms including bacteria and fungi that are capable of breaking down cellulose into monosaccharides either aerobically or anaerobically. The anaerobic bacteria include *Bacteroids cellulosolvents*, *Bacillus* spp. *Clostridium cellulolyticum*, *Clostridium cellulovorans*, *Cellvibrio gilvus*, *Candida lusitance*, etc. The fermentation of cellulose yields a variety of products, e.g., ethanol, lactate, acetate, butyrate, H_2, CO_2, etc.

Introduction of bacteria has been the greatest microbiological innovation because they produce less biomass, low concentration of by-products, and high productivity. The bacterium *Z. mobilis* ferments glucose to ethanol by with a typical yield of 5–10% higher than that of most of the yeasts though it is lesser ethanol tolerant than industrial yeast strains [151]. However, the small bacterium is difficult to centrifuge. *Zymomonas* being a simple prokaryote, an important possibility for the future is development of genetically modified organisms especially tuned to more ethanol tolerance and improved centrifugability [109].

Clostridium thermosaccharolyticum, *Thermoanaerobacter ethanolicus*, and other thermophillic bacteria as well as *Pachysolen tannophilus* yeast [177] are employed in fermenting pentose sugars which are nonfermentable by other organisms usually employed in ethanol production. These bacteria also convert hexose sugars. They have minimum end-product inhibition because very high temperature reactions would allow simple continuous stripping of ethanol from the active fermenting mixture. The yield of alcohol was further improved by coculturing C. *thermocellum* with C. *thermosaccharolyticum* or *C. thermomophydrosulphuricum* [156]. However, the organisms so far studied produce excessive quantities of undesirable by-products and require strict anaerobic conditions which would be difficult to maintain on an industrial scale [53, 154].

Several microorganisms, including bacteria, yeasts, and filamentous fungi, have capacity to ferment lignocellulosic hydrolysates generating ethanol. Among them, *Escherichia coli*, *Z. mobilis*, *S. cerevisiae*, and *P. stipitis* are the most relevant in the context of lignocellulosic ethanol bioprocesses. These microorganisms have different natural characteristics that can be regarded as either advantageous or disadvantageous in processes of ethanol production from hemicelluloses (Table 9.8).

Pure and mixed cultures of *Z. mobilis* and *Saccharomyces* sp. were tested for the production of ethanol by fermentation of medium containing sucrose (200 g/l) at 30°C. The best results were obtained using fermentation for 63 h by a mixed culture and the average hourly ethanol productivity was 1.5 g/l [2, 161]. Ethanol fermentation from culled apple juice was compared by using *Sacharomyces* and *Zymomonas* spp. Ethanol production from culled apple juice showed that fermentability of the juice could be enhanced by addition of Di-ammonium hydrogen phosphate (DAPH) or ammonium sulfate in *Saccharomyces* and DAHP in *Zymomonas*. Trace elements however, inhibited the fermentation in both the cases. Physicochemical characteristics of the fermented apple juices were also analyzed.

Table 9.8 Characteristics of the most relevant microorganisms considered for ethanol production from hemicelluloses

Characteristics	Microorganism			
	E. coli	*Z. mobilis*	*S. cerevisiae*	*P. stipitis*
D-glucose fermentation	+	+	+	+
other hexose utilization (D-galactose and D-mannose)	+	−	+	+
pentose utilization (D-xylose and L-arabinose)	+	−	−	+
Direct hemicellulose utilization	−	−	−	w
Anaerobic fermentation	+	+	+	−
Mixed-product formation	+	w	w	w
High ethanol productivity (from glucose)	−	+	+	−
Ethanol tolerance	w	w	+	w
Tolerance to lignocelluloe derived inhibitors	w	w	+	w
Osmotolerance	−	−	+	w
Acidic pH range	−	−	+	w

+, Positive; −, negative; w, weak

Overall, *S. cerevisiae* proved better than *Zymomonas* for fermentation of apple juice [161].

9.3.4 Fermentation of Syngas into Ethanol

Microorganisms capable of converting syngas into ethanol and other bioproducts are predominantly mesophilic (Table 9.9). The most favorable operational temperature for mesophilic microorganisms is between 37 and 40°C whereas for thermophilic, the temperature varies between 55 and 80°C. Some thermophilic microbes, however, can operate at a higher temperature. The most favourable pH range for efficient microbial activity varies between 5.8 and 7.0, employed to conduct the fermentation, depending upon the species.

9.4 Biochemistry of Fermentation

9.4.1 Fermentation of Carbohydrates

Carbohydrates serve as the chief source of energy in all heterotrophs with supplementation by proteins and fats. The metabolic sequence of energy generation from these major groups of nutrients suggests that carbohydrates are the source of

Table 9.9 Frequently used mesophilic and thermophilic microorganisms, and their optimum growth conditions

Species	Temperature optimum(°C)	pH optimum	Products	References
Mesophilic microorganisms				
Acetobacterium woodii	30	6.8	Acetate	[49]
Butyribbacterium methylotrophicum	37	5.8–6.0	Acetate, Butyrate, Lactate, Pyruvate	[168]
Clostridium aceticum	30	8.5	Acetate	[171]
Clostridium autoethanogenum	37	5.8–6.0	Acetate, ethanol	[3]
Clostridium ljungdahlii	37	6.0	Acetate, ethanol	[184]
Clostridium carboxidivorans	38	6.2	Acetate, ethanol, butyrate, butanol	[113]
Clostridium leatocellum SG6	35	7–7.2	Acetate,lactate, ethanol	[146]
Thermophilic microorganisms				
Moorella thermoautotrophica	58	6.1	Acetate	[164]
Clostridium thermoaceticum	55	6.5–6.8	Acetate	[32]
Clostridium thermocellum	60	7.5–6.0	Acetate	[47]
Carboxydocella sporoproducens	60	6.8	H_2	[173]

energy in the primitive form of life. In the following section, the degradation of carbohydrates, especially polysaccharides that are generally the source of energy liberated either by fermentation or through other metabolic processes, will be discussed.

9.4.1.1 Glucose

Among hexoses, glucose is the immediate metabolizing sugar that can be fermented through different pathways such as glycolysis. The orientation of the -H and -OH groups around the carbon atom adjacent to the terminal primary alcohol carbon (carbon 5 in glucose) determines whether the sugar belongs to the D or L series. When the -OH group on this carbon is on the right side, the sugar is the D-isomer; when it is on the left, it is the L-isomer. Most of the monosaccharides occurring in mammals are D sugars (Fig. 9.1), and the enzymes responsible for their metabolism are specific for this configuration. In solution, glucose is dextrorotatory—hence the alternative name dextrose, often used in clinical practice. Other important hexoses like galactose and mannose are first either converted into

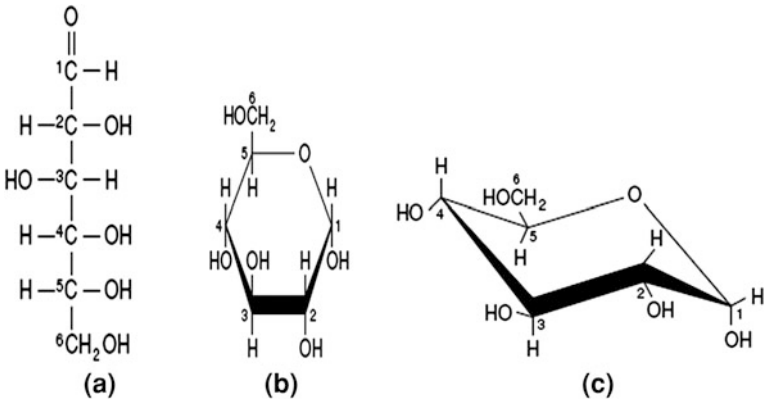

Fig. 9.1 D-Glucose. **a** Straight chain form. **b** α-D-Glucose; Haworth projection. **c** α-D-Glucose; chair form

glucose before fermentation or their products after initial metabolism join the glycolytic sequence. Figure 9.2 shows the pathway of glucose degradation.

9.4.1.2 Sucrose

This disaccharide is most commonly used as the carbon and energy source by fermentative microorganisms. It is a non-reducing sugar consisting of one molecule each of D-glucose and D-fructose linked through α-1, *β*-2 glycosidic bond (Fig. 9.3). In the fermentation process, sucrose is first hydrolyzed by invertase (sucrase) to D-glucose and D-fructose. D-glucose directly enters the glycolysis while fructose joins the main stream after phosphorylation with ATP in a hexokinase-catalyzed reaction. Sucrose can also be fermented through its initial breakdown by sucrose phosphorylase (Fig. 9.4).

9.4.1.3 Lactose

Lactose is a milk sugar. In dairy products, the fermentation of this sugar plays a vital role. Lactose is a disaccharide of D-galactose and D-glucose bonded to each other by *β*-1,4 glycosidic linkage. Lactose cannot be taken up freely by the microbial cells. A specific transport system is required for the translocation of this sugar to the site of metabolism. Lactose transported through PTS gets phosphorylated as lactose-6-P, while the other system translocates it unphosphorylated. Once lactose is translocated, it is fermented first undergoing hydrolysis into monosaccharides with the help of *β*-galactosidase, also called lactase. The former enzyme is present in the lactic acid bacteria. Approximately, 80% of the galactose originated from lactose is metabolized *via* tagatose pathway. Figure 9.5 shows the structure of lactose.

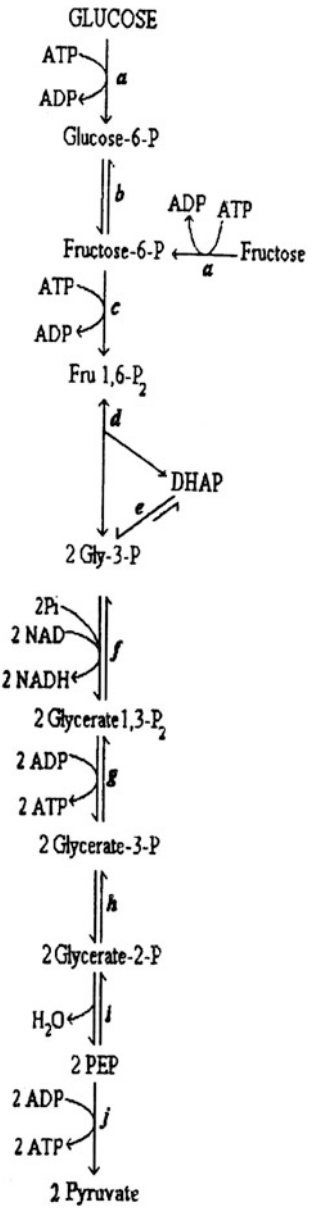

Fig. 9.2 Pathway of glucose degradation. *a* hexokinase, *b* phosphoglucose isomerise, *c* phosphofructokinase, *d* aldolase, *e* triosephosphate, *f* glyceraldehydes-3-P-defydrogenase, *g* phosphoglycerate kinase, *h* phosphoglycerate mutase, *i* enolase, *j* pyruvate kinase

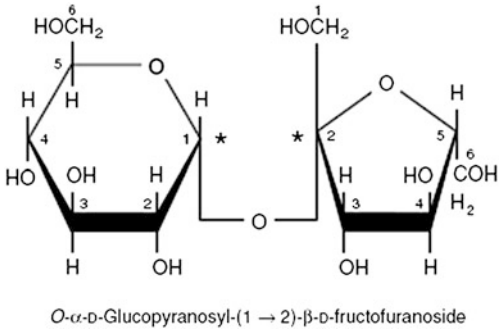

Fig. 9.3 Structure of sucrose

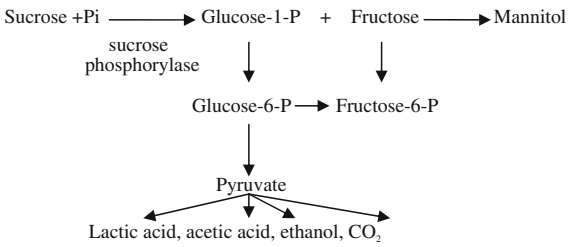

Fig. 9.4 The scheme of sucrose fermentation

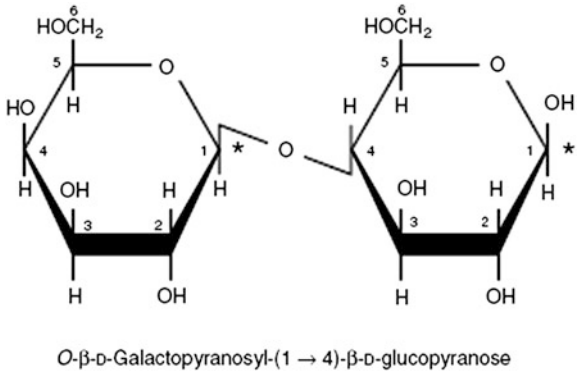

Fig. 9.5 Structure of lactose

9.4.1.4 Starch

Starch is a homopolysaccharide of D-glucose units that are joined to each other through α 1,4-glycosidic bond. Starch has two components, amylose and amylopectin (Fig. 9.6). Amylose is an unbranched molecule with molecular weight ranging from a few thousands to 5,000,00. One end of each chain with free hemiacetal group is reducing while the other is nonreducing in nature. The typical blue color with starch is due to its ability to form a helical structure. It is soluble in water. Amylopectin is a branched polysaccharide with β 1–6 linkage at every

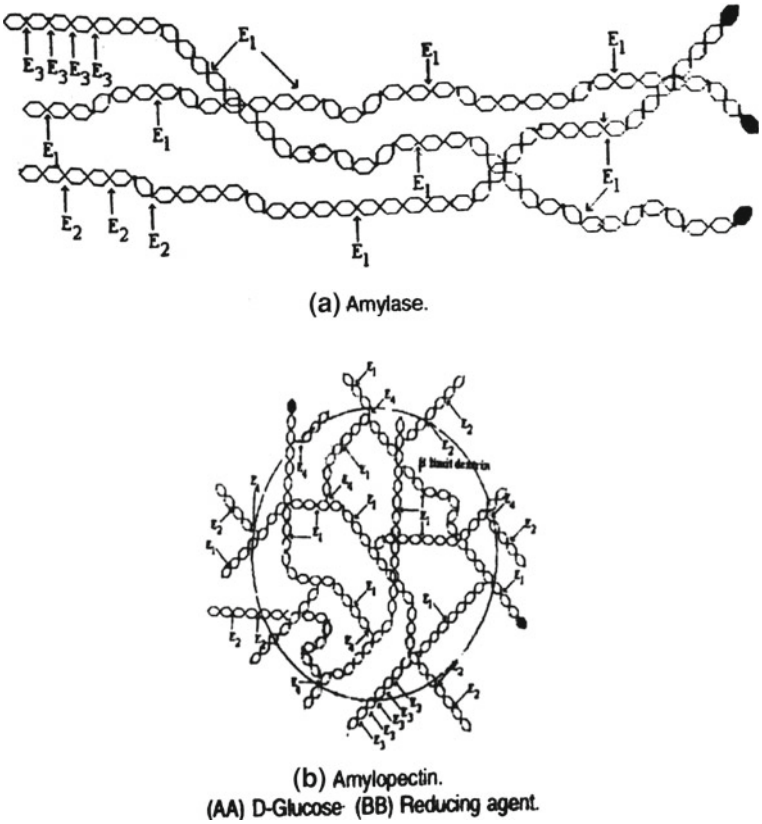

Fig. 9.6 Diagrammatic depiction of action of amylases, starch phosphorylase, and 1→6 glucosidase on starch

25–30 glucose units. The molecular weight and branching per chain differ for different sources of starch.

Starch is widely distributed from lower microalgae such as *Chlamydomonas* to higher plants. In plants, it is the major storage material. A great diversity of microorganisms is able to utilize this polysaccharide. The hydrolysis of starch into glucose in biological systems is carried out with multiple enzymes. For the commercial application of amylolytic enzymes, the reader is referred to an earlier review [62].

9.4.1.5 Cellulose

It is the most abundant material on earth. About 50% of the CO_2 fixed photosynthetically is stored in the form of cellulose [43] as a result of the total

photosynthetic activity [165]. The cereal straw contains 30–40% cellulose while in cotton, flex, etc. the contents are as high as 98%. This form of carbon if recycled can meet the future needs of food energy. Being highly resistant to acid hydrolysis, the recycling process is not without problems. Microorganisms play a pivotal role in recycling of cellulosic carbon. The higher eukaryotes are unable to hydrolyze this polymer. However, the ruminants do so with the help of intestinal microbes.

Cellulose is a homopolysaccharide of D-glucose units joined in a linear fashion through β-1,4-glycosidic linkage (chain length 1.5×10^4 glucose units). The cellulose molecules are joined to each other through hydrogen bonds and *van der wall* forces. The cellulose is insoluble in water and does not give characteristic color with iodine. There is a large number of microorganisms including bacteria and fungi which are capable of breaking down cellulose into monosaccharides either aerobically or anaerobically. The anaerobic bacteria include *Bacteroids cellulosolvents*, *Bacillus* sp., *C. cellulolyticum*, *C. cellulovorans*, *Cellvibrio gilvus*, *Candida lusitance*, etc. The fermentation of cellulose yields a variety of products, e.g., ethanol, lactate, acetate, butyrate, H_2, CO_2 , etc. Due to its water insoluble nature and impermeability to cell wall, the hydrolytic degradation of cellulose occurs through extracellular secretion of enzymes. A single enzyme cannot accomplish the task of cellulose hydrolysis and requires multiple enzymes.

As shown in Fig. 9.7, the saccharification of cellulosic material to glucose involves three types of enzymes: (i) endo-β-1, 4 glucosidase, (ii) exo-cellobiohydrolase, and (iii) β-glucosidase. The activities of both endo-glucanase and exo-cellobiohydrolase are regulated by cellulose through feedback inhibition. The action of β-glucosidase removes cellobiose by hydrolyzing it to glucose that allows the cellulolytic enzymes to function more efficiently. However, β-glucosidase is sensitive to inhibition by its substrate as well as product. A high glucose tolerant β-glucosidase from *Candida* sp. [158] has been purified as efforts to tap cellulosic biomass to form glucose and its subsequent fermentation to ethanol.

9.4.1.6 Hemicelluloses

These are components of cell walls associated with cellulose and are the second largest available organic renewable resource [36]. Hemicellulose consists of xyloglucans with a chain of D-xylose linked through β-1-4 glycosidic bond (Fig. 9.8). The xylose polymer normally contains side chain branches of α-1-3 linked D-mannose and β-1-2 linked D-galactose, β-1-4 linked D-mannose and α-1-2 linked D-glucose. In hardwood hemicelluloses, the xylose units are intermittently esterified with acetic acid at the hydroxyl group of carbon 2 and/or 3 [112].The xylan of softwood, however, is not esterified. The presence of side groups, protruding from the linear β-1, 4 configuration, increases the solubility and thus, renders the substrate easily to hydrolysis.

Due to the complex structure of hemicelluloses, several enzymes are needed for their enzymatic degradation. The main glucanase depolymerizing the hemicellulose

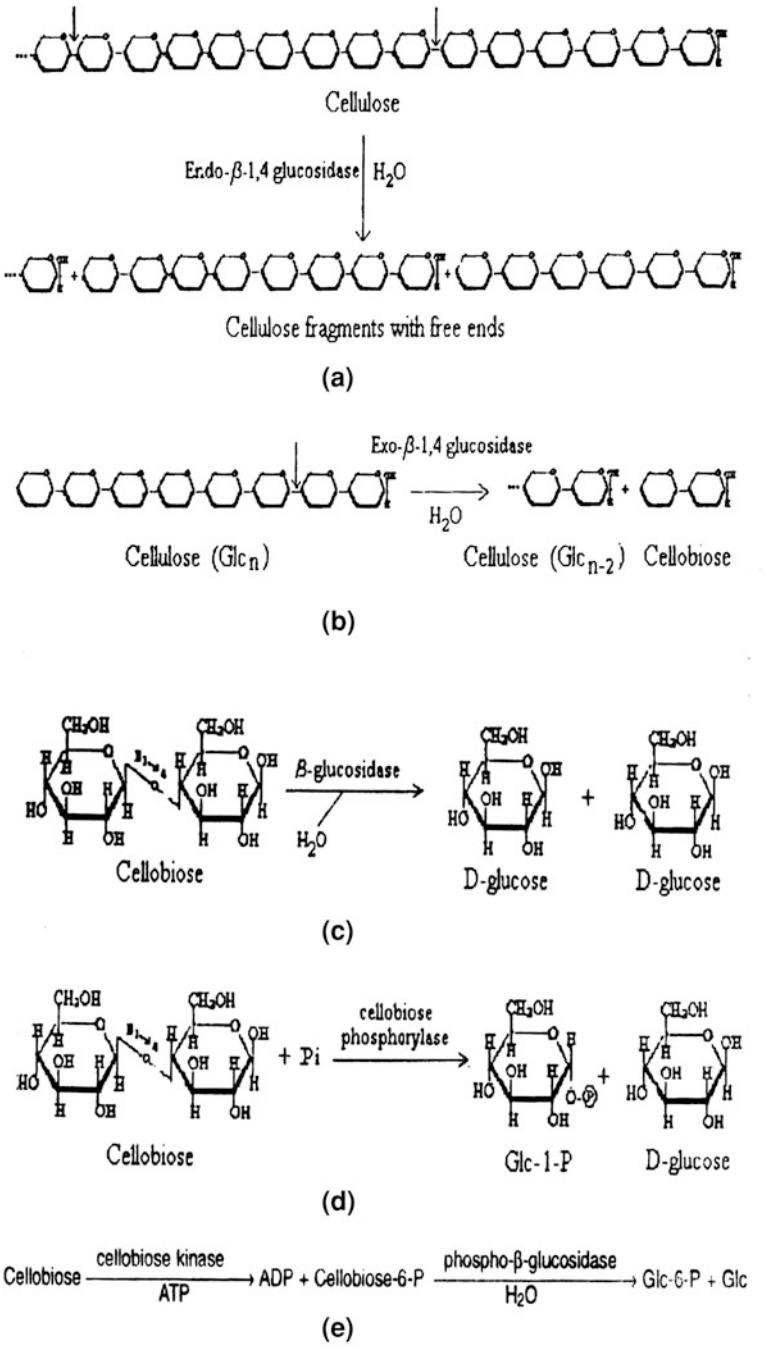

Fig. 9.7 Enzymes involved in cellulose degradation. **a** Endo-β-1,4 glucosidase. **b** Exo-β-1,4 glucosidase. **c** β-glucosidase. **d** Cellobiose phosphorylase. **e** Cellobiose kinase and phospho-β-glucosidase

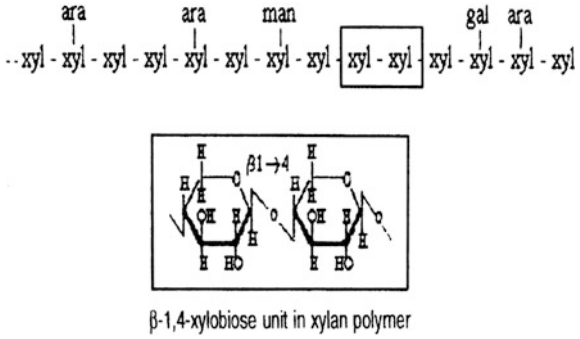

Fig. 9.8 Hemicellulose

backbone is endo-1,4-β-D-xylosidic linkages in xylans resulting in the production of small oligosaccharides. The enzyme does not hydrolyze xylobiose and xylotriose. The xylan-oligosaccharides are further hydrolyzed by the action of exo-1,4-β-D-xylosidase which removes successive D-xylose residues from the non-reducing terminal. The action of xylanase is, however, restricted due to side chains. Nevertheless, the accompanying arabinosidase, galactosidase, glucuronidase, and mannosidase remove the branch points allowing xylanase action. The monomeric xylose molecules are fermented to ethanol or can be utilized to produce single cell proteins or single cell oil [44, 46].

9.4.2 Efficiency of Ethanol Formation

$$\underset{\text{glucose}}{C_6H_{12}O_6} + 2ADP + 2Pi \rightarrow \underset{\text{ethanol}}{2CH_3CH_2OH} + 2CO_2 + 2ATP$$

As shown in the above equation, one molecule of glucose produces two molecules each of ethanol and CO_2, under anaerobic conditions. In other words, 180 g of glucose (1 mol) should yield 92 g of ethanol (2 mol) and 88 g of CO_2 (2 mol). The theoretical yield of ethanol production, therefore, comes to 51%. Under practical conditions, a very high percent (i.e., 47%) yield can be achieved. The metabolism though yields equimolar quantity of CO_2 and ethanol, the actual amount of CO_2 liberated is less than theoretical. This is because of partial reutilization of CO_2 in anabolic carboxylation reactions [138]. According to an estimate, about 85% of the sugars are metabolized to ethanol and CO_2, and the energy produced is used for various cell functions. The rest of the sugars are channeled for biosynthetic reactions. Figure 9.9 shows the pathway of conversion of pyruvate into ethanol and CO_2.

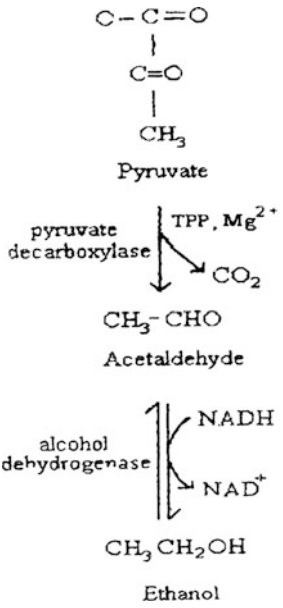

Fig. 9.9 Pathway of conversion of pyruvate into ethanol

9.4.3 Metabolic Engineering for the Production of Advanced Fuels

Use of ethanol as a biofuel has several limitations, such as high vapor pressure, low energy density, and corrosiveness, which prevents its widespread utilization [73, 74, 76, 141]. Bioethanol production, higher alcohols, fatty acid derivatives including biodiesels, alkanes, and alkenes are more compatible with gasoline-based fuels and allow direct utilization. Some of these compounds are also important chemical feedstocks. Since native organisms do not produce these compounds naturally in high quantities, metabolic engineering becomes essential for producing these compounds in non-native producing organisms such as *E. coli*. The four major metabolic systems that allow the production of higher alcohols are the coenzyme-A mediated pathways, the keto acid pathways, the fatty acid pathway, and the isoprenoid pathways which have been discussed in the subsequent sections.

9.4.3.1 The Coenzyme-A-Dependent Fermentative Pathways

n-Butanol and isopropanol are the two higher alcohols which are overproduced in nature by *Clostridium* species. The fermentative pathway in this organism starts from acetyl-CoA. The enzyme acetyl-CoA acetyltransferase condenses two molecules of acetyl-CoA to one molecule of acetyl-CoA. This molecule branches the pathway into isopropanol and n-butanol. For the biosynthesis of isopropanol, an acetoacetyl-CoA transferase transfers the CoA group away from acetoacetyl-CoA

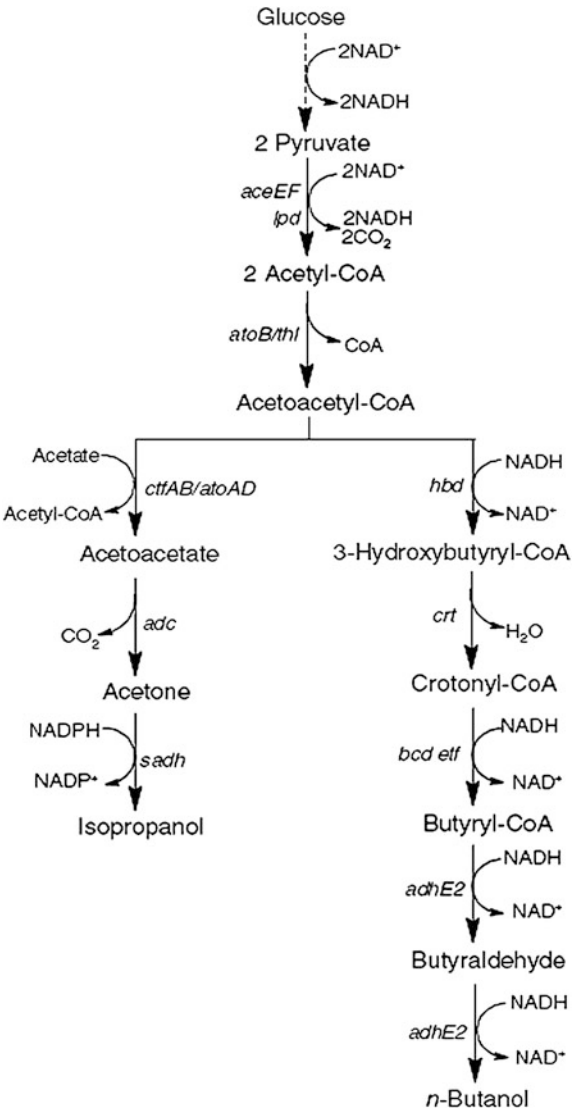

Fig. 9.10 Metabolic pathways for isopropanol and 1-butanol production in engineered *E. coli*. The *dashed line* indicates omitted steps. Isopropanol pathway consists of four enzymatic steps from acetyl-CoA. 1-Butanol pathway consists of six enzymatic steps. *aceEF* and *lpd* encode pyruvate dehydrogenase; *atoB/thl* encodes acetyl-CoA acetyltransferase; *ctfAB/atoAD* encodes *acetoacetyl*-CoA transferase; *adc*, acetoacetate decarboxylase; *sadh* encodes secondary alcohol dehydrogenase; *hbd* encodes 3-hydroxybutyryl-CoA dehydrogenase; *crt* encodes crotonase; *bcd* encodes butyryl-CoA dehydrogenase; *etf* encodes electron transfer Xavoprotein; *adhE2* encodes aldehyde/alcohol dehydrogenase. *Source* [64]

to acetate or butyrate, forming acetoacetate. Acetoacetate is decarboxylated to acetone by an acetoacetate decarboxylase. Then, acetone is reduced to isopropanol by a NADPH-dependent secondary alcohol dehydrogenase [64]. For *n*-butanol biosynthesis, acetoacetate has to go through four steps of NADH-dependent reduction and one step of dehydration as shown in Fig. 9.10.

Isopropanol and *n*-butanol are produced by *Clostridium* species. However, production by this procedure is difficult to handle and optimize, because of

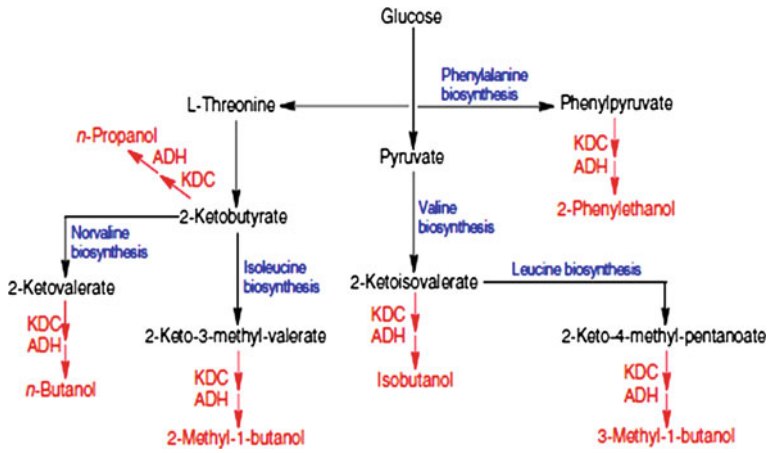

Fig. 9.11 Schematic illustration of higher chain alcohol production via keto acid pathway. keto acid decarboxylase (KDC), alcohol dehydrogenase (ADH)

complex physiological features, such as oxygen sensitivity, slow-growth rate, and spore-forming life cycles of *Clostridium*. Therefore, *E. coli* has been metabolically engineered to produce acetone, the immediate precursor of isopropanol [15] and *n*-butanol production by using the traditional CoA-dependent pathway originated from *C. acetobutylicum* [8].

9.4.3.2 The Keto Acid Pathways

In a heterologous host such as *E. coli*, a non-native pathway introduces non-native metabolites and potential toxicity, difficult to express in heterologous enzymes. Consequently, metabolic imbalance and cytotoxicity that poses as a barrier for large quantity production. It is therefore, necessary to seek for pathways that are compatible with the host. Biosynthesis of amino acid generates many keto acid intermediates. By using decarboxylation and reduction catalyzed by keto acid decarboxylase and alcohol dehydogenase, these keto acids can be converted into alcohols. For example, the isoleucine biosynthesis pathway generates *n*-propanol and 2-methyl-1-butanol, valine biosynthesis pathway produces 2-keto-isovalerate which is the precursor for isobutanol, the leucine biosynthesis generates 2-keto-4-methyl-pentanoate, which is the substrate for 3-methyl-1-butanol, the phenylalanine biosynthesis pathway leads to 2-phenylethanol and nor-valine biosynthesis pathway produces a substrate for *n*-butanol [9].These pathways have been recently used for the production of alcohols in *E.coli* with good results (Fig. 9.11).

9.4.3.3 The Fatty Acid Biosynthesis Pathway

Fatty acid biosynthesis pathway uses acetyl-CoA as a starting molecule [114]. Acetyl-CoA is converted into malonyl-CoA by the addition of a carboxyl group using acetyl-CoA carboxylase as a catalyst. The acetyl and malonyl groups on acetyl-CoA and malonyl-CoA are transferred to a small protein called acyl carrier protein (ACP), which has 77 amino acid residues with a phosphopantothene group specifically attached to a serine residue. Acetyl-ACP and malonyl-ACP are condensed to generate acetoacyl-ACP. This molecule, then goes through reduction, dehydration, and another reduction step to form a 2,3,4-saturated fatty acyl-ACP. The fatty acids synthesized have a long carbon chain backbone, which stores a large amount of energy. To transform fatty acids into combustible fuels, pathways leading to biodiesels and long-chain alkanes/alkenes have been proposed. The fatty acyl-CoA can be reduced to the corresponding fatty aldehydes, which are in turn decarboxylated to long-chain alkanes or further reduced to fatty alcohols that can also be esterified to biodiesel with acetyl-CoA by an alcohol acyltransferase or ester synthase [1, 148]. Biodiesel as a possible substitute for petroleum-based diesel fuel is made from plant oils through transesterification of triacylglycerols with methanol or ethanol. Large-scale application of biodiesel seems difficult because of the seasonal restrictions and the costliness of the transesterification procedure [89]. To overcome these drawbacks, *E. coli* was engineered to produce fatty acid ethyl esters, where the traditional pathway of ethanol consisting of pyruvate decarboxylase (PDC) and alcohol dehydrogenase was introduced to supply ethanol as building units. The metabolically engineered *E. coli* was reported to have capability to produce fatty acid ethyl esters at a titer of 1.28 g/l, by using glucose and oleic acid as substrates.

9.4.3.4 Isoprenoid Pathway

Isoprenoids are natural hydrocarbons biosynthesized for a wide variety of functions. The isoprenoid pathway has been engineered in heterologous hosts to produce nutraceuticals or pharmaceuticals [155]. Despite this, isoprenoids synthesized from isoprenyl diphosphate and dimethylallyl pyrophosphate which are either synthesized from glyceraldehydes-3-phosphate or pyruvate. Recently, two genes in *Bacillus subtilis* 6051 whose products can convert the prenyl diphosphate precursors into corresponding isoprenoids have been reported [195].

9.5 Genetically Modified Microorganisms for Bioethanol Production

Genetical engineering techniques have been applied to increase substrate range in microorganisms such as *S. cerevisiae* and *Z. mobilis* that help to maximize ethanol production like that in *E. Coli*. It also supplies other important traits for conversion

of lignocellulose into ethanol. Since the molecular basis for ethanol and inhibitor tolerance is not fully understood, random mutagenesis and evolutionary engineering have also been applied to improve those traits. Moreover, as a result of technological developments, systems biology approaches have recently been applied to characterize the functional genomics of microorganisms and to evaluate the impact of metabolic and evolutionary engineering strategies. This advanced characterization (genomics, transcriptomics, proteomics, metabolomics) is already contributing to better understand that physiological responses and to identify crucial targets for metabolic engineering [14, 90, 189].

9.5.1 *Escherichia coli*

In *E. coli*, the obvious and successful strategy to increase ethanol production has been the expression of the ethanologenic pathway from *Z. mobilis*, with the genes encoding PDC and ADH II organized in a single plasmid, the PET operon [73, 76], the latter integrated in the chromosome [134]. Subsequent selection of mutants with high ADH activity and disrupted fumarate reductase (for succinate production) originated KO11 strain that produces ethanol at a yield of 95% [135]. However, this strain is unable to grow in ethanol concentrations of 3.5% [199]. Evolutionary genetic engineering strategies were then, applied during a 3-month period, by alternating selection for ethanol tolerance in liquid media and selection for increased ethanol production in solid medium [199]. The resulting strain, LY01, was able to grow in ethanol concentrations of 5%. Coincidentally, this strain became more resistant to aldehydes (including HMF and furfural), organic acids, and alcohol compounds i.e. found in hemicelluloses hydrolysates [201–203]. However, LY01 strain performed poorly in mineral medium compared to rich medium [199]. To avoid dependence on nutritional supplementation, a new strain was produced from SZ110 [200], while the parental strain KO11 was engineered for lactate production in mineral medium [211].

9.5.2 *Zymomonas mobilis*

Contrary to *E. coli*, *Z. mobilis* is an ethanologenic bacterium and lacks the ability to metabolize hemi-celluloses derived monosaccharides, except glucose. Therefore, most of the engineering strategies applied to this bacterium intended to increase their substrate utilization range. In an earlier study, the strain CP4 has been shown to be the best ethanol producer from glucose. It was first engineered toward xylose utilization by the expression, on a plasmid, of the *E. coli* genes encoding for xylose isomerase (XI), xylulokinase (XK), transaldolase (TAL), and transketolase (TKL) under the control of strong constitutive promoters [206]. Ethanol yield from xylose fermentation attained 86% of the theoretical. The same

approach was used to engineer the strain ATCC 39676 toward arabinose fermentation [35]. The genes from the *E. coli* operon araBAD, encoding L-arabinose isomerase (AI), L-ribulokinase (RK), L-ribulose-5P 4-epimerase (L-RPE), together with TAL and TKL allowed L-arabinose fermentation at high yield (96%) but at a low rate. This was ascribed to very low affinity of the glucose facilitator to L-arabinose. The same ATCC 39676 strain was used to express the xylose pathway, followed by successful long-term (149 d) adaptation in continuous fermentation of hemicellulose hydrolysates containing xylose, glucose, and acetic acid [106]. Finally, co-fermentation of glucose, xylose, and arabinose was obtained by genomic DNA integration (AX101 strain) of the xylose and arabinose pathways [124]. The co-fermentation process yield was about 84%, with preferential order in sugar utilization: glucose first, then xylose, and arabinose last.

9.5.3 *Pichia stipitis*

Contrary to *S. cerevisiae*, *P. stipitis* is able to naturally utilize L-arabinose and/or D-xylose and efficiently ferments xylose to ethanol, being the gene donor of the xylose catabolic pathway successfully expressed in *S. cerevisae*. It has also been considered for fermentation of hemicellulose hydrolysates to ethanol [78–80]. Several auxotrophic mutants with higher fermentation capacities and improved xylose utilization have been developed in order to obtain suitable *P. stipitis* strains for further hemicellulose-to-ethanol metabolic engineering [78]. *P. stipitis* is, however, unable to grow anaerobically and is more sensitive to ethanol and inhibitors than *S. cerevisiae*. The *S. cerevisiae* gene that confers the ability to grow under anaerobiosis (URA1, encoding the dihydroorotate dehydrogenase) was successfully expressed in *P. stipitis*, allowing anaerobic fermentation of glucose to ethanol [170]. In addition, the disruption of the cytochrome c gene increased xylose fermentation and consequently, ethanol yield [169]. In an evolutionary engineering approach, *P. stipitis* was adapted in hemicellulose hydrolysate containing glucose, xylose, and arabinose, improving tolerance to acetic acid and pH [131]. In a CBP perspective, xylan conversion into ethanol was enhanced by the heterologous expression of fungal xylanases in *P. Stipitis* [38]. The recent progress in genomic and transcriptomic characterization of *P. stipitis* [80] opened new perspectives for metabolic engineering towards efficient hemicellulose fermentation.

9.5.4 *Kloeckera oxytoca*

Similar to recombinant *E. coli*, ethanologenic strains, *K. oxytoca* M5A1 was engineered with PDC/ADH from *Z. mobilis* for ethanol production from glucose and xylose [135]. The maximal volumetric productivity from xylose was

comparable to glucose and almost twice as that previously obtained with *E. coli* KO11. Stabilization was achieved by chromosomal integration of the heterologous genes [40], allowing the strain to be used in hydrolysates and in simultaneous saccharification and fermentation (SSF) processes. This strain co-ferments glucose, arabinose, and xylose to ethanol, by this order of peference [19]; of notice is the fact that *K. oxytoca* is able to naturally metabolize XOS, as mentioned earlier [145].

9.5.5 *Saccharomyces cerevisiae*

S. cerevisiae is the preferred industrial microorganism for ethanol production because of its excellent fermentability and higher tolerance to industrial conditions. However, *S. cerevisiae* has some problems in producing ethanol from lignocellulosic materials, which are different from that of starch. Hemicelluloses, the second most common polysaccharide in nature, represent about 20–35% of lignocellulosic biomass. However, *S. cerevisiae* cannot utilize pentose released from hemicelluloses of lignocellulosic materials, thus decreasing the yield of ethanol production. In addition, although *S. cerevisiae* is robust, it cannot adequately resist the inhibitors derived from the process of pretreatment of lignocellulose [119].

Pentoses such as xylose and arabinose are the second most abundant fermentable sugars in the hydrolysate from agricultural residues. *S. cerevisiae* cannot utilize them due to the absence of enzymes in the first steps of the metabolic pathways. It is desired for xylose and arabinose to be fermented into ethanol by the industrial *S. cerevisiae* yeast strains to improve ethanol production efficiency and reduce the cost of the production [198].

Metabolic engineering technologies have been widely developed to set up the new pathways in *S. cerevisiae*. Wang [191] constructed the recombinant plasmids containing the genes that encode xylose reductase (XR) and xylitol dehydrogenase (XDH) from *P. stipitis*. Xylulokinase (XK) from *S. cerevisiae* has been transformed into the industrial strain of *S. cerevisiae* for the co-fermentation of glucose and xylose. This recombinant strain NAN-127 consumed twice as much xylose and produced 39% more ethanol than the parent strain in shake-flask fermentation [191]. However, the expression of so many enzymes in a single microorganism may represent a metabolic burden that negatively influences the fermentation capacity [54]. Most of the efforts in lignocellulosic ethanol production with *S. cerevisiae* has been directed to improve the pentose fermentation. The expression of the *P. stipitis* genes XYL1, encoding a xylose reductase (XR), and XYL2, encoding a xylitol dehydrogenase (XDH), was the first successful approach for D-xylose utilization by *S. cerevisiae* [99, 185]. The first recombinant strains produced xylitol from D-xylose rather than ethanol. It was then suggested that the endogenous xylulokinase (XK), encoded by XKS1, could be limiting the performance of *S. cerevisiae* on D-xylose.

9.6 Fermentation

The term 'fermentation' is derived from the Latin verb *fervere*, to boil, thus describing the appearance of the action of yeast on extracts of fruit or malted grain. The appearance of boiling is due to the production of carbon dioxide bubbles caused by the anaerobic catabolism of the sugars present in the extract. However, fermentation has different meanings according to biochemists and to industrial microbiologists. Biochemically, it relates to the generation of energy by the catabolism of organic compounds, whereas its meaning in industrial microbiology tends to be much broader.

In alcoholic fermentation, the substrates that are mainly sugars are fermented, with ethanol as the main product. It is widely distributed among microorganisms. Even plants switch to this pathway for a short period under anaerobic conditions. However, the yeast cell, especially the species of *Saccharomyces* is the main alcohol producer. Some bacteria, particularly *Z. mobilis*, which only utilize hexoses, can also produce ethanol from glucose [150]. In other bacteria, the alcohol is not a predominant end product. Certain yeasts including *S. cerevisiae* can also ferment pentose sugar, xylose to ethanol though the yield is lower compared to the fermentation of hexoses. The production of alcohol by the action of yeast on malt or fruit extracts has been carried out on a large scale for many years and was the first 'industrial' process for the production of a microbial metabolite. Thus, industrial microbiologists have extended the term fermentation to describe any process for the production of a product by the mass culture of a microorganism. It may be noted that the fermentation equipment makes upto 10–25% of the total fixed capital cost of an ethanol plant depending upon its design.

9.6.1 Fermentation Kinetics

9.6.1.1 Yeast Metabolic Pathways

Glucose is converted into ethanol and CO_2 *via* glycolysis, in the anaerobic pathway:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \text{Energy (Stored as ATP)}$$

The overall reaction produces two moles of ethanol and CO_2 for every mole of glucose consumed, with the reaction energy stored in 2 mol of ATP. Every gram of glucose converted will yield 0.511 g of ethanol, *via* this pathway. Secondary reactions consume a small portion of the glucose feed, however, to produce biomass and secondary products, Pasteur found that the actual yield of ethanol from fermentation by yeast is reduced to 95% of the theoretical maximum (Table 9.10). For maximum ethanol productivity, aerobic reaction should be avoided as in this

Table 9.10 Optimum yields from anaerobic fermentation by yeast

Product	g per 100 g glucose
Ethanol	48.4
Cabon dioxide	46.6
Glycerol	3.3
Succinic acid	0.6
Cell mass	1.2

Source: [71]

reaction, sugar is completely converted into CO_2, cell mass and by-product with no ethanol formed.

9.6.1.2 Effect of Sugar Concentration

The primary reactant in the yeast metabolism is hexose sugar (glucose, fructose). The rate of ethanol production is related to the available sugar concentration by a Monod-type equation under fermentative conditions:

$$V = V_{max}C/(K_s + C_s),$$

where

V = specific ethanol productivity (g ethanol/g cells/h)

C_s = Sugar substrate concentration (/g)

K_s = Saturation constant having a very low value (typically 0.2–9.4 g/l).

The yeast is starved at very low substrate concentrations (below 3 g/l) consequently, the productivity decreases [105]. At higher concentrations, a saturation limit is reached so that the rate of ethanol production per cell is essentially at its maximum up to 150 g/l sugar concentration. The catabolic (sugar) inhibition of enzymes in the fermentative pathway becomes important above 150 g/l, and the conversion rate is slowed down [72, 192].

An important secondary effect of sugar is catabolic repression of the oxidative pathways—*Crabtree Effect*. At above 3–30 g/l sugar concentration (depending on the yeast strain), the production of oxidative enzymes is inhibited [34, 127] thus, fermentative pathway is adopted. The Crabtree effect is not found in all the yeasts and is a desirable character in the industrial strains of yeast selected.

9.6.1.3 Effect of Ethanol

Ethanol is also inhibitory to the microorganisms producing it. It has three inhibitory effects: inhibition of cell multiplication, inhibition of fermentation, and a lethal effect on cells (Table 9.11). It is toxic to yeasts and high bioethanol tolerances capacity of yeast is a pre-requisite for production of bioethanol. It has been

Table 9.11 Effect of bioethanol concentration (P) on specific growth rate (u) of some yeasts in batch culture

Saccharomyces cerevisiae NRRL-Y-132		*Saccharomyces cerevisiae* ATCC 4126		*Saccharomyces cerevisiae* NCYC-479	
Pu (/g)	$u(h^{-1})$	Pu (/g)	u (h^{-1})	Pu (/g)	$u(h^{-1})$
0	0.4	0	0.44	0	0.280
24	0.264	50	0.36	20	0.251
50.4	0.17	60	0.36	40	0.200
66.0	0.091	80	0.28	60	0.139
80.2	0.043	100	No growth	80	0.018
90	No growth			100	0.024

Source [9, 128]

shown that the inhibitory effect of ethanol is generally negligible at low concentrations (less than 20 g/l) but increases rapidly at higher concentrations [13]. For most strains, ethanol production and cell growth are stopped completely at above l00 g ethanol/l although some very slow fermenting yeasts *(Saccharomyces sake)* can tolerate higher ethanol concentrations at low temperatures [23, 70]. Ethanol inhibition is directly related to the inhibition and denaturation of important glycolytic enzymes as well as to the modification of the cell membrane [123, 153]. Various factors, viz., temperature, aeration, medium composition, etc. influence bioethanol sensitivity directly or indirectly, modify the properties of cell membrane, and membrane lipids.

9.6.1.4 Effect of Oxygen

Aerobic metabolism which leads to utilization of sugar substrate but produces no alcohol must be avoided to a great extent. However, the trace amounts of oxygen may greatly stimulate yeast fermentation. Oxygen is required for yeast growth as a building block for the biosynthesis of polyunsaturated fats and lipids required in mitochondria and plasma membrane [69]. High sugar concentration is adequate to repress aerobic sugar consumption in yeasts which shows the Crabtree effect. For other yeasts or at low sugar concentrations, the oxygen supply should be limited. Trace amounts of oxygen (0.7 mm Hg Oxygen tension) are adequate and do not promote aerobic metabolism [30].

9.6.1.5 Effect of pH

Fermentation rate is sensitive to pH, but most distiller's yeasts show a broad pH optima from 4 to 6 [29]. Most yeast strains are capable of tolerating high acidic pH (2) in the solutions without any permanent damage [71].

9.6.1.6 Effect of Temperature

High temperature tolerance is a desirable quality selected for distillery yeasts and most distillery yeasts have a temperature growth optima of 30–35°C [56]. For low alcohol concentrations, the optimum fermentation temperature is slightly higher (up to 38°C) but alcohol tolerance is improved at reduced temperatures [70]. Exposure to temperatures above the optimum results in excessive enzyme degradation and loss of yeast viability. Yeast metabolism liberates 11.7 KCal of heat per kg of substrate consumed [103]. Yeast is inactive at low temperature (0°C) and can be stored at that temperature and readily revived [178].

9.6.1.7 Additional Nutrient Requirements

Mash must be enriched with secondary nutrients in addition to the sugar source for ethanol production. Secondary nutrients are necessary for cell maintenance and growth [82]. Yeast extract NH_4C1, $MgSO_4$, $CaCl_2$ are a few of the ingredients which promote very rapid cell growth and ethanol production at laboratory scale [30, 31]. Ammonium ions provide nitrogen for protein and nucleic acid synthesis. Yeast extract contains all the necessary yeast growth factors viz., amino acid, purines, pyrimidines, vitamins, and minerals. Phosphorous, potassium (from yeast extract), magnesium, and calcium are incorporated into cell mass and are also cofactors activating several enzymes. The wide variation in media compositions used for different yeasts for alcohol production resulted in different yields.

Several organic and inorganic nitrogen sources in media for ethanol production by *Z. mobilis* were tested [176]. Urea and yeast extract were found to be better sources and calcium pantothenate was found to be an essential vitamin for ethanol production.

9.6.1.8 Secondary Component Inhibition

Fermentation by-products or non-metabolized feed components can inhibit the ethanol production and yeast growth. These secondary components become more concentrated when used and this limits the recycling process of distillery residue.

Acetate and lactate are the most important inhibitory fermentation by-products [125]. Certain inhibitors are high in a few substances, e.g., sulfite waste liquor may be high in sulphurous acid and furfural. Blackstrap molasses may contain high concentrations of calcium salts. High temperature, sugar concentration, and sterilization in the presence of salts (especially phosphates) and proteins can produce components toxic to yeast [22].

When important individual inhibitors are not present, a combination of inhibitors or generalized osmotic pressure effects shall be the limiting factors. High salt concentrations also encourage the production of undesirable by-products such as

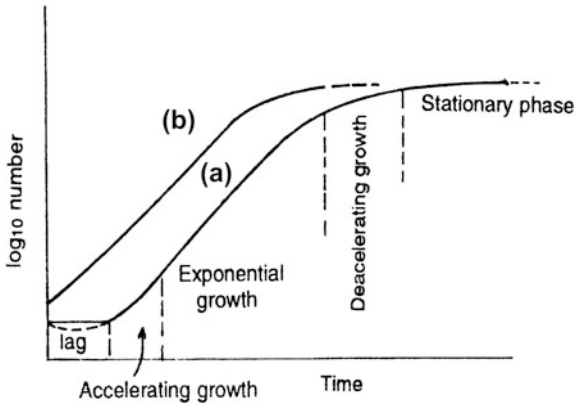

Fig. 9.12 Growth of unicellular organisms in batch culture. **a** Normal growth with lag phase. **b** Growth without lag phase

glycerol [193]. A 16–20% non-fermentable dissolved solids content sets the practical upper limit for most yeasts in the absence of toxic inhibitors [52].

9.6.2 Fermentation Process for Bioethanol

9.6.2.1 Conventional Batch Fermentation

Batch cultures are simple, closed systems. In this system, all the substrates are added at the beginning, before inoculation, and neither anything is added or taken out during the fermentation. A typical growth curve is followed by the organism (Fig. 9.12a) in this type of fermentation. In industrial processes, generally, the actively growing inoculum is added to avoid any lag phase as it leads to the wastage of time (Fig. 9.12b) The batch fermentation has certain limitations like exhausting of nutrients, accumulation of antagonists, product inhibition, etc. which eventually affects the product formation.

The product is recovered at the end of the growth phase. This involves emptying the fermenter out and processing the medium to get the product out. The fermenter has to be cleaned, refilled, resterilized, and then, reinoculated. Such operations are called *turn-round* and the time it takes to do it is called *down time*.

Figure 9.13 depicts the batch fermentation equipment layout incorporating heat exchangers and chemical sterilization systems. Most of the currently practiced alcohol fermentations are based on the traditional processes described above. But many advanced methods have been developed in order to increase the productivity, reduce the capital investment, and better utilization of energy. Such advances are the use of continuous fermentations, the increase of yeast population by recycling, and the removal of ethanol during fermentations.

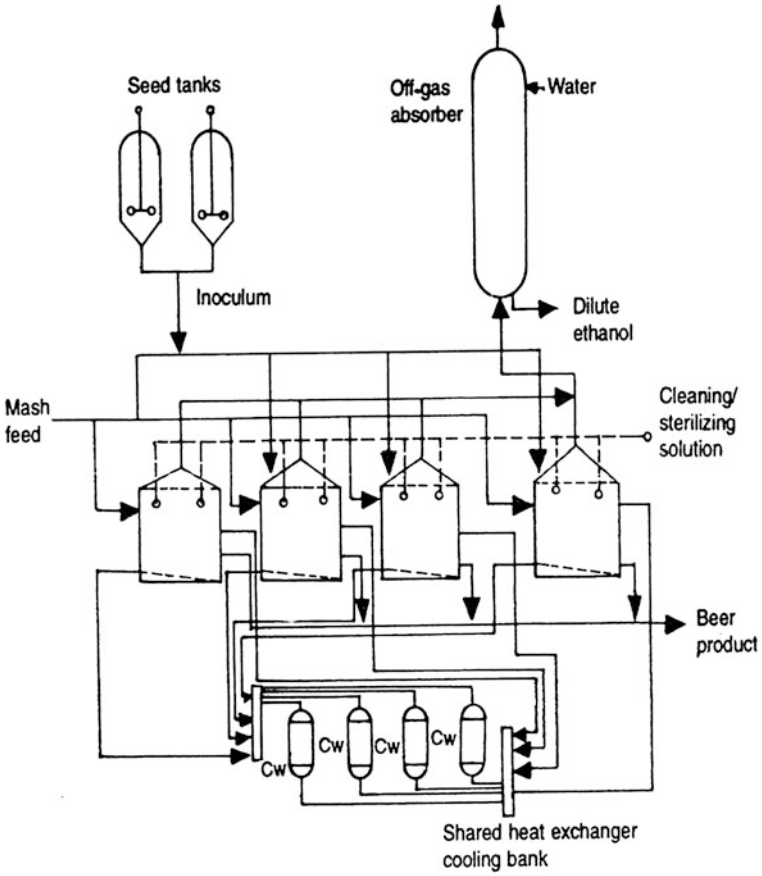

Fig. 9.13 Batch fermentation equipment layout incorporating teamed heat exchanger and chemical sterilization systems. *Source* [52]

9.6.2.2 Continuous Fermentation

In continuous fermentation, fresh medium is continuously pumped into the fermenter and an equal volume of the fermented liquid is continuously pumped out for recovery of ethanol and yeast. This is an open system. The rate at which medium is added or at which the fermenter liquid is withdrawn is expressed as the dilution rate D which is the ratio of withdrawn liquid (F) to the volume of total liquid in the fermenter (V) i.e. $D = F/V$ (Units of D are h^{-1}).

Feed is pumped continuously into the fermenter displacing beer which then overflows from the vessel. The composition of the produced beer is the same as the composition inside the fermenter. Therefore, the fermenter is to run at a relatively slow rate to obtain a higher concentration of alcohol because it will allow complete utilization of sugar and growth of new yeast cells in the fermenter to replace

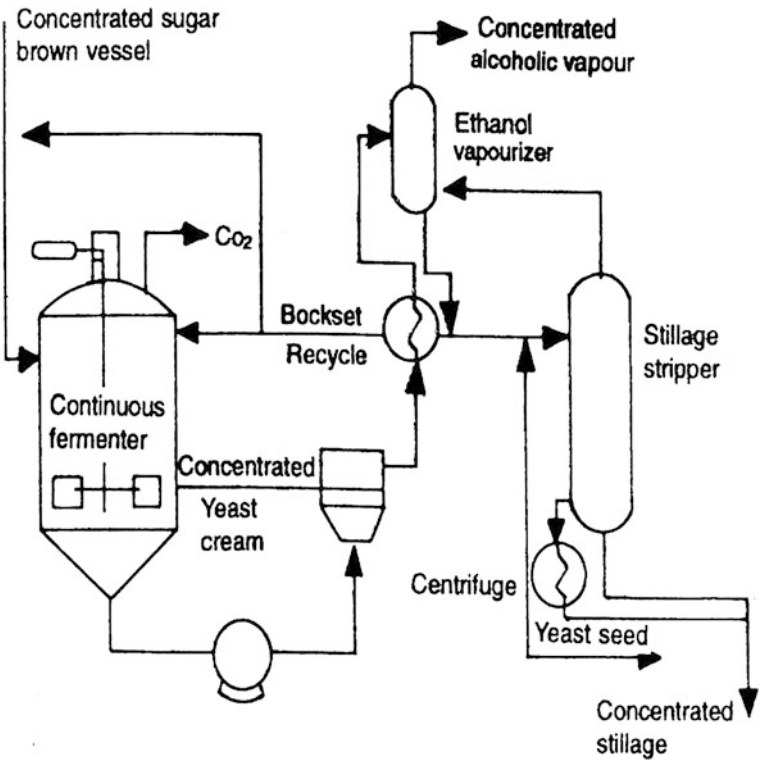

Fig. 9.14 Biostil fermentation process. *Source* [52]

washed out cells [139]. Stirring is an important factor for successful continuous flow fermentation. The modification of the continuous fermentation process is the Biostil process (Fig. 9.14).

9.6.2.3 Fed-Batch Fermentation

A variable volume fed-batch culture was adopted (incremental feeding of same concentration solution to that of initial medium resulting in an increase in volume). All the fermentations were performed in a fed-batch mode in a 5-l bioreactor controlled by a computer having advanced fermentation soft water. The fermenter was equipped with temperature, agitation, and aeration systems with precise control for these parameters. Aeration was measured in terms of dissolved oxygen. The parameters were measured and automonitored against the set values. The pH was, however, controlled manually by adding acid or alkali as the case may be. The volume of incremental feeding was adjusted in such a way that the final volume in the fermenter reached to about 4.75–5.00 l. The samples were drawn

using sampling port at a 2-h interval during fermentation using injection syringe under aseptic conditions. Incremental feeding was started after 1 h of actual start of fermentation (called as activation period) and stopped before 1 h of actual completion of fermentation (called as terminal cell maturity step). For incremental feeding additional accessories were attached to the fermenter. Generally, 7–8 h were taken by incremental feeding at this rate. The fermentation parameters were kept arbitrary but constant, except that used for standardization during the parameter optimization experiments.

9.7 Technology of Bioethanol Production

Bioethanol can be produced from the processing industry waste rich in sugar/starch by the microbial technology that may evolve an alternative to our limited and non-renewable resource of energy. Increasing environmental regulations for controlling waste disposal will further enhance the possibilities of ethanol production from waste.

9.7.1 Sugar Molasses

A process has been developed for the preparation of power alcohol from molasses on pilot scale with immobilized whole cells. Ethanol production from molasses has also been scaled up with addition of 15% total sugar content using *Z. mobilis* [39]. A scheme of fuel ethanol production from sugarcane bagasse has been shown in Fig. 9.15. Ethanol production by *Z. mobilis* can be increased by addition of calcium carbonate in high sugar medium and at higher fermentation temperature (43°C) [175].

Batch fermentations of sugarcane blackstrap molasses to ethanol using pressed yeast as inoculum, demonstrated an exponential relationship between the time necessary to complete fermentation and the initial concentrations of sugar and the yeast cells [18]. Fed-batch alcoholic fermentation of sugarcane blackstrap molasses (at 32°C, pH 4.5–5.0) without air and compressed yeast enhanced the average yeast yields and average yeast productivities without affecting the ethanol yield.

Neutral spirits and ethanol are the major fermentation products from citrus molasses [21, 51]. In Florida only, 1 million L of alcohol is produced from citrus molasses annually. The process includes dilution of molasses to 25°B followed by fermentation yeast. The alcohol is recovered by distillation. Enzymatic digestion of citrus peel, solubilizing of 85% total peel solids with 65% hexose sugar [133] made available more sugar for fermentation, thus increasing the yield of alcohol. However, reduced yield of alcohol has been reported from molasses produced by

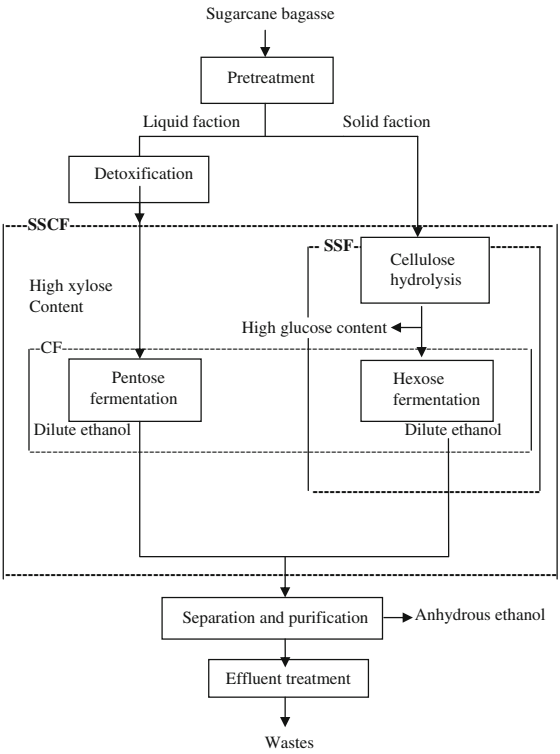

Fig. 9.15 Process of fuel ethanol production from sugarcane bagasse. Possibilities for reaction–reaction integration are shown inside the shaded boxes: CF, cofermentation; SSF; SSCF, simultaneous saccharification, and cofermentation

heat evaporators (30–50°B) where some loss of fermentable sugar during handling and storage might have taken place [21].

9.7.2 Apple Pomace

Traditionally, alcohol is produced from liquid or liquid mash *via* submerged microbial fermentation. In recent years, there has been a considerable interest in the production of alcohol from food processing wastes such as apple pomace because of (i) the rising energy costs of molasses and (ii) the negative cost of values of wastes as substrates. Apple pomace is not readily amenable to submerged microbial fermentation due to its nature. The solid-state fermentation of apple pomace offers several advantages for ethanol production such as higher yield but has difficulty of ethanol extraction from the solid materials. Different microorganisms (Table 9.12) have been used for the production of ethanol,

Table 9.12 The various microorganisms used for apple pomace fermentation with ethanol yield and fermentation efficiency

Micro-organisms	Ethanol yield (%)	Fermentation efficiency (%)	References
Saccharomyces cerevisiae	2.86–4.31	70.0–94.0	[63]
Saccharomyces cerevisiae	3.7–5.4	44.5–64.9	[60]
Saccharomyces diasticus	3.6–5.7	43.3–68.5	[60]
Saccharomyces cerevisiae	3.92–4.3	60.0–68.0	[88]
Candida utilis	3.71–4.59	57.0.69.0	[88]
Torula utilis	3.93–4.10	59.0–62.0	[88]

predominantly yeast belonging to *S. cerevisiae* that has been a microorganism of choice.

Hang [66] developed a solid-state fermentation system of apple pomace with *S. cerevisiae* at 30°C in 96 h producing 43 g ethanol/kg of apple pomace. Ethanol was separated out by vacuum evaporation with a separation efficiency of 99%. Blending the pomace with molasses lowered the ethanol yield and fermentation efficiency. However, fermentation by immobilized yeast did not increase the yield of ethanol from the apple pomace. Jarosz [77] collected apple pomace from three factories and fermented at 30°C for 72 h with or without addition of inoculum. The natural microflora induced the fermentation but addition of yeast accelerated the fermentation and brought to the 78.9% of the theoretical yield of ethanol.

Sandhu and Joshi [160] reported that natural fermentation of apple pomace was inferior to the yeast inoculated fermentation for ethanol, crude, and soluble proteins. The production of ethanol in natural fermentation was almost half that of *S. cerevisiae* fermentated apple pomace. Joshi et al. [85, 88] provided partial aseptic and anaerobic condition to the solid-state fermentation of apple pomace by addition of SO_2 and found that addition of SO_2 up to 200 ppm increased the ethanol content by *S. cerevisiae* while it was 150 ppm for *Candida utilis* and *Torula utilis*. The amount of ethanol present in the fermented apple pomace depends upon the initial sugar content in the apple pomace which in turn is influenced by variety of apple processed, the processing conditions, and the amount of the pressing aids employed.

Ethanol recovery by manual squeezing, direct distillation of fermented pulp, percolation of fermented pulp and hydraulic pressing in three stages with interstate water addition from solid-state fermented pulpy material have revealed that hydraulic pressing in three stages with interstate water addition, led to 79.68% ethanol recovery with 60.53% ethanol in the pooled extract of that in the fermented pulp. Ngadi and Correia [130] found that when the apple pomace was fermented at 77 and 85% of moisture level yielded 19.26 and 18.10% of ethanol on dry weight basis. The original pH and the initial moisture content of apple pomace was found to be suitable for ethanol production, decreasing the pH or increasing the moisture content reduced the ethanol content [87]. Fermentation time increased the ethanol production up to 96 h at 30°C and among the different nitrogen sources tried,

ammonium sulfate gave the highest ethanol production and *S. cerevisiae* giving better response to it than *Candida utilis* and *Torula utilis*. Addition of 0.4% of ammonium sulfate increased the ethanol yield. The combined effect of AMS and $ZnSO_4$, however, was detrimental to ethanol production but AMS alone gave better ethanol yield.

Gupta [61] found that the addition of nitrogen, phosphate, and trace elements to the SSF of apple pomace with *Saccharomyces diasticus* enhanced the fermentation efficiency to 67.7, 68.5, and 68.8%, respectively (control having fermentation efficiency of about 43.8%). Distillation of fermented extract with a bucchi evaporater yielded 0.029, 4.1, 0.0003, 0.01, and 0.011% of methyl, ethyl, n-propyl, isobuty1, and isoamyl alcohols, respectively [66]. Joshi and Sandhu [83] found that all the yeast fermented apple pomace distillates contained methyl and butyl alcohols and aldehyde. *S. cerevisiae* fermented distillate had more desirable characteristics than those obtained from fermentation with other yeasts and thus, had potential for conversion into potable alcohol. The step-by-step process involved in ethanol production from food processing industry waste is shown in Fig. 9.16.

9.7.3 Orange Waste

Orange waste coming from food industries is used in continuous fermentation. It has been found that fixed bed immobilized cell reactor showed maximum ethanol production [50]. Use of citrus processing by-product mainly peel by fermentation by *S. cerevisiae* for ethanol production has been reported [58, 94]. The initial saccharification of polysaccharides by commercial cellulase and polygalacturonase followed by removal of inhibitory compounds by filtration and pH adjustment of the hydrolysate was necessary for successful fermentation [29].

Ethanol has also been produced from lignocellulosic waste by employing recombinant bacterial strains of *E. coli* and *Klebsiella oxytoca* [91]. The bacterial strains had the capacity to produce ethanol from pentose sugars. The conversion of monosaccharides in orange peel hydrolysates into ethanol by recombinant *E. coli* (KOll) was in pH controlled batch fermentations that led to very high yields of ethanol. The microorganism was capable of converting all major monosaccharides in orange peel hydrolysates into ethanol and to a smaller amount of acetic and lactic acids [57]. Citrus molasses prepared by evaporation and concentration of the press liquor and molasses mixed with the citrus pulp have also been used by distillaries as an alcohol feedstock [50]. Initial moisture content of the solid medium has been shown to be a limiting factor for maximum ethanol production [130]. Industrial alcohol has also been produced from waste fruits such as apple, pear, and cherry through fermentation [11].

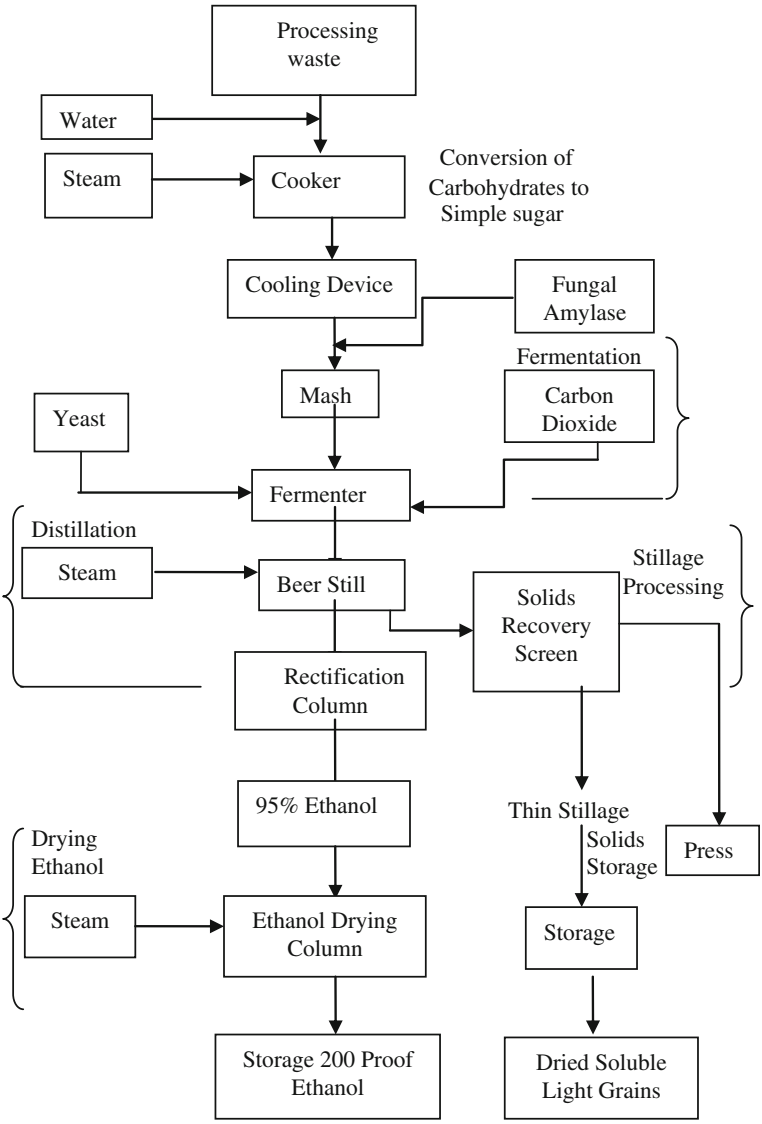

Fig. 9.16 Flow diagram of the process involved in ethanol production

9.7.4 Banana Waste

Recently, ethanol production potential of waste bananas has been assessed [63]. Ethanol yield from normal banana was found to be as: ripe whole fruits 0.091, pulp 0.082, and peel 0.0061/kg of whole fruits. The green fruit gave 0.090, normal ripe

0.082, and overripe 0.0691/kg of ethanol. Enzymatic hydrolysis was necessary for higher ethanol yield while dilution with water was not essential for effective fermentation.

9.7.5 Potato Waste

The use of potato peel waste for the production of alcohol has also been made [17]. The acidified peel waste (pH 6) is used for ethanol production.

9.7.6 Wheat Straw

Wheat straw like any other biomass of lignocellulosic composition is a complex mixture of cellulose, hemicellulose, and lignin, as three main components, and a small amount of soluble substrates (also known as extractives) and ash. The cellulose strains are bundled together and tightly packed in such a way that neither water nor enzyme can penetrate through the structure [104]. Hemicellulose serves as a connection between lignin and cellulose fibers, and it is readily hydrolyzed by dilute acid or base, as well as hemicellulase enzyme. Lignin is covalently linked to cellulose and xylan (predominant hemicellulose carbohydrate polymer in wheat straw) such that lignin–cellulose–xylan interactions exert a great influence on the digestibility of lignocellulosic materials [104]. Due to this, the structural complexity of the lignocellulosic matrix, ethanol production from wheat straw requires at least four major unit operations including pretreatment, hydrolysis, fermentation, and distillation. Unlike sucrose or starch, lignocellulosic biomass such as wheat straw need to be pretreated to make cellulose accessible for efficient enzymatic depolymerization.

9.7.7 Rice Straw

Rice straw is one of the most abundant lignocellulosic crop residues in the world. The worldwide availability of rice straw and theoretical ethanol yield is shown in Table 9.13. Technologies for conversion of this feedstock into ethanol have been developed on two platforms, which can be referred to as the sugar platform and the synthesis gas (or syngas) platform. In the sugar platform, cellulose and hemicellulose are first converted into fermentable sugars, which are then fermented to produce ethanol. The fermentable sugars include glucose, xylose, arabinose, galactose, and mannose. Hydrolysis of cellulose and hemicellulose to generate these sugars can be carried out using either acids or enzymes [41].

Table 9.13 Worldwide availability of rice straw and theoretical ethanol yield

Country	Rice straw availability (million MT)	Theoretical ethanol yield (billion l)
Africa	20.93	8.83
Asia	667.59	281.72
Europe	3.92	1.65
North America	10.95	4.62
Central America	2.77	1.17
South America	23.51	9.92

Source [95]

9.7.8 Rice Husk

Possibilities of the utilization of rice husk and subsequent chemical conversion of hemicellulose into xylose, followed by furfural, xylitol, xylonic acid, and ultimately the food yeast is explored [55]. Similarly, hydrolysis of cellulose to glucose which, then, can be converted into ethanol, sorbitol, hydroxy methyl furfural, levulinic acid, etc. is outlined. However, ethyl alcohol production would be economical only if all the by-products are recovered and processed. Mucilagenos material from cocoa waste is another source of alcohol [129]. The waste from tapioca spent pulp after concentration by centrifugation to 20% solids after hydrolysis holds promise for production of alcohol.

9.7.9 Barley

The waste from a novel, vacuum distillation procedure (30–45°C) called Mugi (Barley) contained a large number of viable yeast (7×10^6 cells/ml), with glucoamylase (19.7 units/ml), acid protease (940 units/ml), and neutral protease (420 units/ml). The waste was mixed with mash composed of glucose as the sole source of carbon. After distillation of fermentation broth, the non-volatile residues were again used in the next ethanol fermentation and the cycle was repeated successfully ten times. The system is developed for the distillery waste which is treated as per the conventional waste water [186].

9.7.10 Whey

Using lactose hydrolyzing yeast under anaerobic conditions, whey can be converted into alcohol [137]. The system though made primarily for SCP production from whey, can also be employed for production of alcohol. Prehydrolyzed whey with β-galactosidase enzyme in which most of the lactose is hydrolyzed has been

used as a substrate for alcohol production. Since the alcohol produced is taxed in a similar manner as the potable alcohol for use in the beverage industry, this proposition also becomes expensive [117]. Such alcohol for use as industrial alcohol or alcohol as a chemical should be taxed at different rates than used for potable beverage production.

9.7.11 Cassava Roots

Cassava roots are used as raw materials for the production of ethanol in some countries like Brazil. The alcohol produced from cassava roots was used as motor fuel, mixed with gasoline (upto 20% alcohol) for which no motor modification is required. It is also used as pure anhydrous ethanol, in which there is need to modify the carburetor and some other parts. Both result in less atmospheric pollution than the use of 100% gasoline. Commercial production of ethanol from cassava is obviously not new in some parts of Asia like India and China. In China, several factories are now using solid waste (bagasse) of the cassava starch industry for the production of ethanol [59].

The suitability of extractive fermentation as a technique for improving the production of ethanol from lactose by *Candida pseudotropicalis* over the conventional technique has also been examined [81, 82]. Using Adol 85 NF, extractive solvent, biocompatible with microorganisms, extractive fed-batch and conventional fed-batch systems were operated for 160 h and the extractive system showed a 60% improvement in lactose consumption and ethanol production with 75% volumetric productivity.

In the syngas platform, the biomass is subjected through a process called gasification. In this process, the biomass is heated with no oxygen or only about one-third the oxygen normally required for complete combustion. It subsequently converts into a gaseous product, which contains mostly carbon monoxide and hydrogen. The gas, which is called synthesis gas or syngas, can be fermented by specific microorganisms or converted catalytically into ethanol. In the sugar platform, only the carbohydrate fractions are utilized for ethanol production, whereas in the syngas platform, all the three components of the biomass are converted into ethanol [41].

9.7.12 Hydrolysed Cellulosic Biomass

Lignocellulose biomass, including wood waste, agricultural waste, household waste, etc. represents a renewable resource which has stored solar energy in its chemical bonds [120]. It has great potential for bioethanol production, when compared to ethanol produced from grain, tubers, and sugar plants, because it is a widely available cheap feedstock which does not compete with human food products.

9.7.12.1 Pretreatment

It is known that the main difficulty in converting lignocellulose biomass into second-generation ethanol consists in breaking down structural and chemical biomass complex. In the course of the breakdown process, cellulose feedstock is affected by enzymes which allow further recovery of ethanol. Biomass consists of polysaccharides-cellulose and hemicellulose, which are hydrolyzed into single sugar components, followed by further recovery of ethanol by well-known and elaborated fermentation technologies. Enzymatic activity in lignocellulose hydrolysis gives a good yield and minimum amount of by-products; it has lower energy consumption, milder operating conditions, and represents an environmentally friendly processing method [157, 194]. Considering that the sugars required for fermentation are bound to the lignocellulose structure, pretreatment of biomass is required in order to remove and/or modify lignin and hemicellulose matrix before enzymatic hydrolysis of polysaccharides. Unlike starch which is a crucial source of energy in plants, cellulose has mostly a structural role as it provides plant cells with mechanical durability with hemicellulose and lignin. Natural cellulose materials do not have high reactivity; therefore, fermentable saccharification requires a large cellulose surface and broken cellulose microfilm structure. Reactivity of natural substrates is also reduced by lignin.The most commonly applied methods can be classified into two groups: chemical hydrolysis (dilute and concentrated acid hydrolysis) and enzymatic hydrolysis. In addition, there are some other hydrolysis methods in which no chemicals or enzymes are applied. For instance, lignocellulose may be hydrolyzed by thermal treatment, wet-oxidation, gamma-rays or electron-beam irradiation, or microwave irradiation. However, these processes are commercially unimportant.

9.7.12.2 Chemical Hydrolysis

In chemical hydrolysis, pretreatment and hydrolysis may be carried out in a single step. There are two basic types of acid hydrolysis processes commonly used: dilute acid and concentrated acid, each with variations.

Acid Hydrolysis

Acid-catalyzed process can be divided into two general approaches, based on concentrate acid/low temperature and dilute-acid/high temperature hydrolysis. Sulfuric acid is the common acid employed although, however, hydrochloric, nitric and trifluoracetic acids, phosphoric acid, weak organic acids have also been used.

Concentrated-Acid Hydrolysis

Concentrate acid processes enable the hydrolysis of both hemicelluloses and cellulose. The solubilization of polysaccharides is reached using different acid concentrations, like 72% H_2SO_4, 41% HCl or 100% TFA [45]. Concentrate-acid-based processes have the advantage to allow operating at low/medium temperatures leading to the reduction in the operational costs. Hydrolysis of cellulosic materials by concentrated sulphuric or hydrochloric acids is a relatively old process. The concentrated acid process uses relatively mild temperatures, and the only pressures involved are those created by pumping materials from vessel to vessel. Reaction times are typically much longer than for dilute acid. This method generally uses concentrated sulphuric acid followed by a dilution with water to dissolve and hydrolyze or convert the substrate into sugar and provides a complete and rapid conversion of cellulose into glucose and hemicelluloses into 5-carbon sugars with little degradation. The critical factors needed to make this process economically viable are to optimize sugar recovery and cost-effectively recovery of the acid for recycling. The solid residue from the first stage is dewatered and soaked in a 30–40% concentration of sulphuric acid for 1–4 h as a pre-cellulose hydrolysis step. The solution is again dewatered and dried, increasing the acid concentration to about 70%. After reacting in another vessel for 1–4 h at low temperatures, the contents are separated to recover the sugar and acid. The sugar/acid solution from the second stage is recycled to the first stage to provide the acid for the first-stage hydrolysis. The primary advantage of the concentrated acid process is the potential for high sugar recovery efficiency. The acid and sugar are separated *via* ion exchange and then, acid is re-concentrated via multiple effect evaporators. The low temperatures and pressures employed allow the use of relatively low cost materials such as fiberglass tanks and piping. The low temperatures and pressures also minimize the degradation of sugars. Unfortunately, it is a relatively slow process and cost- effective acid recovery systems have been difficult to develop. Without acid recovery, large quantities of lime must be used to neutralize the acid in the sugar solution. This neutralization forms large quantities of calcium sulfate, which requires disposal and creates additional expense. Moreover, the equipment corrosion is an additional disadvantage. Nevertheless, there seems to be a renewed interest in these processes [209] owing to the moderate operation temperatures and because no enzymes are required.

Dilute-Acid Hydrolysis

Pretreatment by using dilute-acid processes for the hydrolysis of hemicellulose renders the cellulose fraction more amenable for a further enzymatic treatment, but in this case a two-step-hydrolysis is required. The dilute acid process is conducted under high temperature and pressure, and has a reaction time in the range of seconds or minutes, which facilitates continuous processing. The difference between these two steps is mainly the operational temperature, which is high in the second step (generally around 230–240°C) [108, 196, 197]. Example cited by using a dilute acid process with 1% sulfuric acid in a continuous flow reactor at a

residence time of 0.22 min and a temperature of 510 K with pure cellulose provided a yield of over 50% sugars. In this case, 1,000 kg of dry wood would yield about 164 kg of pure ethanol. The biggest advantage of dilute acid processes is their fast rate of reaction, which facilitates continuous processing.

Compared to the concentrate acid hydrolysis, one of the advantages of dilute-acid hydrolysis is the relatively low acid consumption, limited problem associated with equipment corrosion, and less energy demanding for acid recovery. Under controlled conditions, the levels of the degradation compounds generated can also be low. As an alternative to the conventional dilute-acid processes, the addition of CO_2 to aqueous solutions, taking advantage of the carbonic acid formation has been described [190], but the results obtained were not interesting enough to consider application.

Alkali Hydrolysis

The use of alkaline pretreatments is effective depending on the lignin content of the biomass. Alkali pretreatments increase cellulose digestibility and they are more effective for lignin solubilization, exhibiting minor cellulose and hemicellulose solubilization than acid or hydro-thermal processes [24]. Alkali pretreatment can be performed at room temperature and times ranging from seconds to days. It is described to cause less sugar degradation than acid pretreatment and it was shown to be more effective on agricultural residues than on wood materials [100]. In alkali hydrolysis possible loss of fermentable sugars and production of inhibitory compounds must be taken into consideration to optimize the pretreatment conditions. Sodium, potassium, calcium, and ammonium hydroxides are suitable alkaline pretreatments. NaOH causes swelling, increasing the internal surface of cellulose and decreasing the degree of polymerization and crystallinity, which provokes lignin structure disruption from 24–55% to 20% [101, 182]. The example of alkali hydrolysis cited below by using Lime pretreatment $Ca(OH)_2$ removes amorphous substances such as lignin, which increases the crystallinity index. Lignin removal increases enzyme effectiveness by reducing non-productive adsorption sites for enzymes and by increasing cellulose accessibility [96]. Lime also removes acetyl groups from hemicellulose reducing steric hindrance of enzymes and enhancing cellulose digestibility [126]. Lime has been proven successfully at temperatures ranging from 85 to 150°C and for 3–13 h with corn stover or poplar wood [27]. Pretreatment with lime has lower cost and less safety requirements compared to NaOH or KOH pretreatments and can be easily recovered from hydrolysate by reaction with CO_2 [126].

Enzymatic Hydrolysis

Enzymatic hydrolysis has an upper edge over acid hydrolysis to produce sugars for alcohol fermentations. Enzymes are naturally occurring plant proteins that cause

certain chemical reactions to occur. There are two technological developments: enzymatic and direct microbial conversion methods. The chemical pretreatment of the cellulosic biomass is necessary before enzymatic hydrolysis. The first application of enzymatic hydrolysis was used in separate hydrolysis and fermentation steps. Enzymatic hydrolysis is accomplished by cellulolytic enzymes. Different kinds of "cellulases", i.e., endoglucanases, exoglucanases, glucosidases, and cellobiohydrolases are commonly used [75, 107] to cleave cellulose and hemicellulose. The endoglucanases randomly attack cellulose chains to produce polysaccharides of shorter length, whereas exoglucanases attach to the non-reducing ends of these shorter chains and remove cellobiose moieties, glucosidases hydrolyze cellobiose, and other oligosaccharides to glucose [142]. In order to enhance the susceptibility of cellulose for enzymatic hydrolysis, the pretreatment of cellulosic material is, therefore, an essential prerequisite. Physical and chemical pretreatments like ball milling, irradiation, alkali treatment, acid treatment, hydrogen peroxide treatment are highly recommended to enhance saccharification of cellulosic material after their enzymatic hydrolysis [6, 167].

So far, cellulose has been hydrolyzed with enzyme cellulase only at pilot plant scale. The process is divided into many steps and includes two basic inputs, namely, nutrients for the fungus and cellulosic material to be hydrolyzed. The nutrients supply include nitrogen and other supplements required for the growth of celluloytic microorganisms and is given in the form of sterilized nutrient medium. Cellulosic materials are pretreated. The celluloytic microorganism is grown and subsequently the enzyme is produced. The microorganism (such as fungus) is propagated as a submerged culture in a fermentation unit equipped for mixing and aerating the growth medium.

In the cellulose hydrolysis or saccharification step, the enzyme produced in the previous step comes into contact with the pretreated cellulosic materials. The enzyme solution hydrolyzes the solid cellulose to the glucose units. The product stream is continuously withdrawn from the unit. Finally, the glucose solution is separated from unhydrolyzed cellulose by filtration. The glucose solution can be used for fermentation to ethanol.

The rate and extent of enzymatic hydrolysis is affected by the pretreatment method, substrate concentration and accessibility, enzyme activity, and reaction conditions such as pH, temperature and mixing [121, 181]. Different strategies for enzymatic hydrolysis and ethanolic fermentation have been developed to address specific process engineering issues (Table 9.14).

Advantages of Biological Pretreatment over Chemical Treatment

Biological pretreatment offers some conceptually important advantages such as low chemical and energy use. However, a controllable and sufficiently rapid system has not yet been found. At the same time, chemical pretreatments have also serious disadvantages in terms of the requirement for specialized corrosion resistant equipment, extensive washing, and proper disposal of chemical wastes.

Table 9.14 Selected hydrolysis and fermentation strategies

Name	Description	Features
SHF: separate hydrolysis and fermentation	Enzymatic hydrolysis and fermentation done sequentially in different vessels	Hydrolysis and fermentation at respective optimal conditions; enzyme product inhibition; separate treatment of C5 and C6 sugar streams
SSF: simultaneous saccharification and fermentation	Enzymatic hydrolysis and fermentation done simultaneously in same vessel	Compromise in conditions for optimal hydrolysis and fermentation; improved rates and yields; separate treatment of C5 and C6 sugar streams
HHF: hybrid hydrolysis and fermentation	Enzymatic hydrolysis and fermentation done roughly sequentially in same vessel	Hydrolysis continues after shift to fermentation conditions; process optimization difficult; separate treatment of C5 and C6 sugar streams
NSSF: non-isothermal simultaneous saccharification and fermentation	Enzymatic hydrolysis and fermentation done roughly simultaneously in different vessels	Hydrolysis and fermentation at respective optimal conditions; process optimization difficult; separate treatment of C5 and C6 sugar streams
SSCF: simultaneous saccharification and co- fermentation	Like SSF, only both C5 and C6 sugars are fermented in same vessel	Fewer vessels, lower capital costs; requires engineered microorganism optimized for efficient C5/C6 fermentation
CBP: consolidated bioprocessing	Enzymatic hydrolysis and fermentation carried out in single vessel by single or combination of microorganisms	Fewer vessels, lower capital costs; requires engineered microorganism optimized for enzyme production and C5/C6 fermentation

Biological pretreatment is a safe and environmentally friendly method for lignin removal from lignocellulose. Biological pretreatment comprises of using microorganisms such as brown, white, and soft-rot fungi for selective degradation of lignin and hemicellulose out of which white-rot fungi seems to be the most effective microorganism. Lignin degradation occurs through the action of lignin-degrading enzymes such as peroxidases and laccase [136]. Biological pretreatments are safe, environmentally friendly, and less energy intensive compared to other pretreatment methods (Table 9.15). However, the rate of hydrolytic reaction is very low and needs a great improvement to be commercially applicable. Hatakka [68] investigated the pretreatment of wheat straw using 19 white-rot fungi and found that 35% of the wheat straw was converted to reducing sugars after 5 weeks' pretreatment with *Pleurotus ostreatus* compared to only 12% conversion of the untreated straw.

Table 9.15 Advantages and disadvantages with different methods for pretreating lignocellulosic biomass

Pretreatment method	Advantages	Disadvantages
Biological	Degrades lignin and hemicellulose Low energy consumption	Low rate of hydrolysis
Milling	Reduces cellulose crystallinity	High power and energy consumption
Steam explosion	Causes lignin transformation and hemicellulose solubilization, Cost-effective, Higher yield of glucose and hemicellulose in the two-step method	Generation of toxic compounds, Partial hemicellulose degradation
Diluted acid	Less corrosion problems than concentrated acid, Less formation of inhibitors	Generation of degradation products, Low sugar concentration in exit stream
Concentrated acid	High glucose yield, Ambient temperatures	High cost of acid and need to be recovered, Reactor corrosion problems, Formation of inhibitors
Organosolv	Causes lignin and hemicellulose hydrolysis	High cost Solvents need to be drained and recycled
Ozonolysis	Reduces lignin content, Does not imply generation of toxic compounds	High cost of large amount of ozone needed
Wet oxidation	Efficient removal of lignin, Low formation of inhibitors, Minimizes the energy demand (exothermic)	High cost of oxygen and alkaline catalyst
CO_2 explosion	Increases accessible surface area, Cost-effective, Do not imply generation of toxic compounds	Does not affect lignin and hemicelluloses, Very high pressure requirements

Thermal Pretreatment

Thermal pretreatment for fractionation and solubilization studies of lignocellulosic materials have shown the efficiency to improve the yields of extraction of hemicelluloses. Boussarsar [20] have evaluated the SCB conversion by hydrothermal treatment. Optimal conditions were 170°C for 2 h, reaching higher solubilization of hemicellulose than that at 150°C and lower degradation of sugar monomers than at 190°C. However, analysis of thermal hydrolysates shows the presence of xylan oligomers and polymers with large chains. On the other hand, Sendelius [166] has evaluated the steam pretreatment conditions with respect to final ethanol yield, using SCB as feedstock. The variables considered were temperature (180, 190, and 205°C), time (5 and 10 min), and impregnating agents (water, 2% SO_2 by weight of water in the bagasse and 0.25 g H_2SO4 per 100 g dry matter). The most prominent tested pretreatment condition was: SO_2-impregnation at a temperature of 180°C during 5 min, which gave a glucose yields in average 86.3% and xylose yields in average 72.0%. The fermentation of these hydrolyzed materials gave an overall ethanol yield of 80%, based on theoretical value.

Wet Oxidation

Wet oxidation (WO) is the process of treating material with water and either air or oxygen at temperatures above 120°C. Two types of reactions occur during WO: a low temperature hydrolytic reaction and a high temperature oxidative reaction. It has been demonstrated that a combination of alkali and WO reduces the formation of toxic furfuraldehydes and phenol aldehydes [97]. Martín [118] have investigated different conditions pH, temperature, and reaction time of WO pretreatment on fractionation and enzymatic convertibility of SCB, while pressure (12 bar) was kept constant. The highest cellulose content, nearly 70%, was obtained in the pretreatment at 195°C, 15 min and alkaline pH. The highest sugar yield in the liquid fraction, 16.1 g/100 g, was obtained at 185°C; 5 min and acidic pH. Although the analysis of the solid fraction in most of the pretreatments showed high degrees of hemicelluloses solubilization, the content of free sugars in the liquid fraction was very low. It is known that WO mainly catalyzes the transfer of hemicelluloses from the solid phase to the liquid phase, but it does not catalyze the hydrolysis of the liberated hemicelluloses molecules. The products of hemicelluloses hydrolysis during WO are not monosaccharides, but sugar oligomers.

9.7.13 Recent Advances in Bioethanol Production Process

Ethanol can be produced in two different ways, either by Direct Microbial Conversion (DMC) [180] or by Simultaneous SSF process. Novel bioreactors consisting of more than one bioreactor along with genetic recombination techniques are being developed at laboratory and pilot scale to improve the yield and productivity of bioethanol [25, 102]. Thermophilic fermentation seems to be a promising technique [122]. Additionally, the use of supercritical CO_2 as a pretreatment option has increased the ethanol yield by 70% [207].

9.7.14 Boiethanol Refinery

The conversion of by-products into value added products under a biorefinery concept may further reduce the associated process costs with additional energy in the form of fuels, heat, and electricity such as formation of xylitol from xylose, methyl fuorate from furfural and plastic from hydroxylmethyl furfural. Nevertheless, estimation of greenhouse gas emissions of these products as they are shaped into marketable products is required. The main technological issues have been summarized recently by Kumar [102]. Prasad [144] described the pros and cons of various pretreatment options for ethanol production from lignocellulosic biomass. Moreover, the availability of the feedstock and related logistics influence the effectiveness of bioethanol technology [180].

9.8 Future Perspectives and Conclusions

An increased use of biofuels would contribute to sustainable development by reducing greenhouse gas emissions and the use of non-renewable resources. In recent years, it has been suggested that instead of traditional feedstocks, cellulosic biomass (cellulose and hemicellulose), including sugarcane bagasse could be used as an ideally inexpensive and abundantly available source of sugar for fermentation into transportation fuel ethanol. The efficiency of biomass conversion into ethanol depends upon the ability of the microorganism used in the process to utilize these diverse carbon sources and the amount of fraction present in biomass. The cost of ethanol production from sugarcane bagasse is relatively high based on current technologies.

As the price of current ethanol feedstocks (e.g. Corn) is estimated to increase, lignocellulosic materials remain the only viable candidate to serve as renewable feedstock for ethanol production. There are huge amounts of wheat straw that are currently burnt in the field or wasted otherwise which can be used as low value raw material for ethanol production. Despite extensive technological advances in ethanol production from lignocellulose feedstocks over the last few decades, the price of the second-generation ethanol is still high and remains around $2.65/gallon [101, 102]. This high price is because of some technological impediments encountered in all the different steps of the process. Pretreatment is estimated to account for 33% of the total cost [187]. The current leading pretreatment methods for lignocellulosic materials are capital intensive. Economical comparison showed that there is little differentiation between studied pretreatment methods as for instance; low cost pretreatment reactors are counterbalanced by higher cost of catalyst and/or ethanol recovery [42]. Development of less energy intensive and more effective pretreatment methods allowing lower amount of enzymes loading can substantially decrease the total cost of cellulosic ethanol.

The utilization of lignocellulosic biomass for bioethanol production necessitates the production technology to be cost-effective and environmentally sustainable. Considering the evolution and need of second-generation biofuels, rice straw appears to be a promising and potent candidate for production of bioethanol due to its abundant availability and attractive composition. Biological conversion of rice straw into fermentable sugars, employing hydrolyzing enzymes is, at present the most attractive alternative due to environmental concerns. Although there are several hindrances in the way of developing economically feasible technology due to its complex nature, high lignin, and ash content, work is going on to develop an efficient pretreatment method to remove unwanted portions so as to get readily available sugars and a considerable success has been achieved till date. The available statistics show that the need of bioethanol for the transport sector could be met by using rice straw. Approaches in both process engineering and strain engineering still have to be carried out to circumvent the difficulties of xylose and glucose co-fermentation and to improve the system efficiency. A very balanced and intelligent combination of pretreatment, hydrolysis, and the fermentation

process has to be selected for maximum efficacy of the process. With the advent of genetically modified yeast, synthetic hydrolyzing enzymes, other sophisticated technologies and their efficient combination, the process of bioethanol production employing rice straw will prove to be a feasible technology in the very near future.

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