BIOETHANOL PRODUCTION

PRINCE SONGKLA UNIVERSITY 2021
VIRTUAL VISITING PROFESSOR PROGRAM

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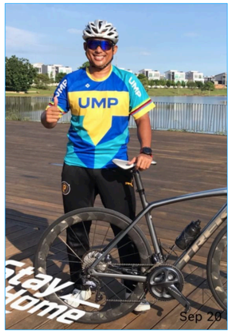

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PRESENTATION OUTLINES

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1.0 INTRODUCTION TO BIOETHANOL

- Bioethanol has significant potential as an alternative fuel that can contribute to fulfil the growing energy demand, especially in the transport sector, and at the same time reduce the carbon dioxide emissions.
- In general bioethanol can be produced through fermentation reaction from starch or sugar-based feedstocks.
- *Saccharomyces cerevisae* or mainly called baker yeast played important role in fermentation because of establishment and widely used in industrial application for wine and beer fermentation.
- *Saccharomyces cerevisae* converts glucose to bioethanol very efficient where it is relatively inexpensive, highly available and can be grown rapidly in two conditions which are aerobic and anaerobic.
- One molecule of glucose, breaks down to give two molecules of ethanol and two molecules of CO_2.
- Applying molecular weight, 180g glucose produces 92g ethanol and 88g CO_2.

$$C_6H_{12}O_6 \xrightarrow{S.\ cerevisae} 2\,C_2H_5OH + 2\,CO_2$$

sugar (e.g.: glucose) → bioethanol + carbon dioxide

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- Conventionally, bioethanol has been produced from starch or sugar-based feedstocks (edible agricultural crops and products) known as 1st generation bioethanol.
- However this feedstock (substrate) conflicts with food and feed production.
- As an alternative to 1st generation bioethanol, currently the 2nd generation bioethanol much focus on advancing a cellulosic bioethanol concept that utilizes lignocellulosic residues from agricultural crops and residues (such as bagasse, straw, stover, stems, and leaves).
- Our class today will be focusing on these two bioethanol generations together with fermentation aspects (upstream), separation and purification processes (downstream).
- We will conclude our class on the future direction of bioethanol production especially on the 3rd and 4th generation bioethanol.

- The world is mainly dependent on non-renewable energy sources for transportation and generation of heat and power.
- The major energy source is fossil fuel as it provide about 78.4% of the final energy consumption worldwide (Renewables, 2017).
- Because of the increasing energy requirements and impacts of fossil fuels use on health and environment, there is an urgent requirement to explore other options (Hussain et al., 2017).
- Depending on the current usage, the discovery rate of fossil fuels, shortly, will not match the utilization rate (Du et al., 2016).
- Biofuels emit lesser greenhouse gases and are a promising source for replacing fossil fuels.
- In addition, biofuels are produced from common biomass sources which are geographically more consistently distributed as compared to fossil fuels, allowing for an autonomous and secured supply of energy (Liew et al., 2014; Nigam and Singh, 2011).

- The use of bioethanol as an alternative fuel has been steadily increasing around the world which can can decrease dependence on foreign oil, reduce trade deficits, reduce air pollution, and reduce global climate change carbon dioxide build up.
- Bioethanol, unlike gasoline, is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NOx emissions from combustion.
- Renewable Fuel Association (RFA) (2017) reported that the blending of ethanol in gasoline, reduced carbon dioxide-equivalent green house gas emissions from transportation by 43.5 million metric tonnes in 2016.
- This is equivalent of removing 9.3 million cars from the road for the whole year (RFA, 2017).
- Brazil and the U.S. are the first bioethanol producing countries.
- In the U.S., the main raw material used is corn starch whereas in Brazil, sugarcane juice is the major raw material used.

- At present, there are more than 200 biorefineries in the U.S., having the capacity of producing about 60.64 billion litre of ethanol per year jointly (RFA, 2017).
- There are 16 biorefineries in Brazil that produce bioethanol from both sugarcane juice and corn starch.
- In EU, Sweden, France, Germany, Italy and the UK are jointly producing more than 2 billion litre of bioethanol annually (Robak and Balcereket, 2018).
- More than 98% of gasoline in the U.S. contains some ethanol with the most common blend of ethanol is E10 (10% ethanol, 90% petrol), to oxygenate the fuel, which reduces air pollution.(U.S. Energy Department, 2021).
- Ethanol is also available as E85 (or flex fuel)—a high-level ethanol blend containing 51% to 83% ethanol, depending on geography and season—for use in flexible fuel vehicles.
- Another blend, E15 is increasing its market presence and is approved for use in model year 2001 and newer light-duty conventional petrol vehicles.

- The use of bioethanol in spark ignition engines shows several benefits when comparison is made with petrol.
- Ethanol has a higher oxygen content, which encourages better combustion and reduced emission of exhaust gases, and a higher octane number.
- This would allow engine to operate at a higher compression ratio (CR) (Bajpai, 2021).
- A higher CR is beneficial for engines because the higher ratio allows for an engine to extract more energy from the combustion process due to better thermal efficiency.
- A higher CR allows the same combustion temperatures to be achieved with less fuel.
- The engine is able is turn more of the heat generated from the combustion process into horsepower instead of wasted heat.
- In addition, the use of agriculture biomass as feedstock (substrate) for bioethanol production would allow for recycling the carbon dioxide emitted during combustion and reduce the carbon dioxide emissions.

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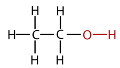
Ethanol



Structural formula

C_2H_5OH

Molecular formula

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Parameter	Unit	Gasoline	Ethanol
Lower calorific value	kJ/kg	43,500	28,225
	kJ/litre	32,180	22,350
Density	kg/litre	0.72 – 0.78	0.792
RON *(Research Octane Number)*	–	90 – 100	102 – 130
MON *(Motor Octane Number)*	–	80 – 92	89 – 96
Vaporization latent heat	kJ/kg	330 – 400	842 – 930
Stoichiometric relation air/fuel		14.5	9.0
Steam pressure	kPa	40 – 65	15 – 17
Ignition temperature	ºC	220	420
Solubility in water	% in volume	~ 0	100

Source: API (1998) and Goldemberg and Macedo (1994).

- Research octane number (RON), a rating used to measure a fuels knocking resistance in spark-ignition internal combustion engines.
- RON is determined by running the fuel in a test engine with variable CR under controlled conditions.
- What is knocking? Knocking is what happens when parts or all of the air-fuel mixture prematurely ignites before the flame from the spark plug can reach it.
- This can be caused by ignition timing that is too early or engine overheating, where the heat from the cylinder itself causes the mixture to combust before the spark plug can burn the mixture.
- Results in decrease in engine performance.

YouTube https://www.youtube.com/watch?v=ltjVT1wyUuw

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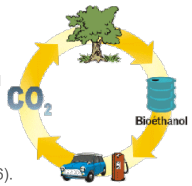
- The interest in bioethanol is the possibility of obtaining a substantial reduction of noxious exhaust emissions from combustion, especially as statutory limits are becoming more stringent.
- Wider use bioethanol will mean that there are fewer harmful effects on life and ecosystems.
- Using bioethanol in place of petrol helps to reduce CO_2 emissions by up 30–50% given today's technology.
- Bioethanol does not add to global CO_2 levels because it only 'recycles' CO_2 already present in the atmosphere.
- CO_2 is released to the atmosphere during combustion.
- CO_2 gets removed from the atmosphere through photosynthesis when crops intended for conversion to bioethanol are grown.
- In reverse, burning a fossil fuel such as petrol adds to global CO_2 as it releases new amounts of CO_2 that were earlier trapped underground for millions of years (EIA, 2006).



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2.0 GLOBAL BIOETHANOL PRODUCTION

- Ethanol has three major uses: as a biofuel, as a beverage, and for industrial purposes.
- About 95% of all ethanol is derived by fermentation from sugar or starch crops; the rest is produced synthetically.
- The bioethanol production routes from sugar, starch and lignocellulosic biomass are based on fermentation or hydrolysis.
- The synthesis route evolves dehydration of hydrocarbons (e.g., ethylene) or by reaction with sulphuric acid, to produce ethyl sulphate, followed by hydrolysis.
- The most common feedstock for bioethanol production comes from starches such as corn, wheat, and potatoes (U.S.), sucrose or sugarcane juice (Brazil), and sugar beets (Europe).
- Lignocellulosic biomass is being used in developing methods, which includes wood, grasses, and agriculture crop residues.
- It is considered developing because converting the cellulose into glucose is more challenging than in sugars and starches.

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Annual World Fuel Ethanol Production (Mil. Gal.)

Region	2015	2016	2017	2018	2019	2020	% of World Production
United States	14,807	15,413	15,936	16,091	15,778	13,926	53%
Brazil	7,200	6,750	6,650	7,990	8,590	7,930	30%
European Union	1,360	1,360	1,420	1,450	1,370	1,250	5%
China	770	670	800	770	1,000	880	3%
India	190	280	200	430	510	515	2%
Canada	450	460	460	460	520	428	2%
Thailand	310	340	390	390	430	400	2%
Argentina	220	240	290	290	280	230	1%
Rest of World	393	487	454	529	522	500	2%
Total	25,700	26,000	26,600	28,400	29,000	26,059	

*Source: RFA analysis of public and private data sources

•Currently, the U.S. produces the most ethanol worldwide (63,308.849 Mil. Lit. - 53%), whereas Brazil is the next largest producer (36,050.49 Mil. Lit. - 30%) with total 118,466.56 Mil. Lit. of bioethanol production worldwide.

•Other countries, including EU (5,682.613 Mil. Lit. - 5%), China India, Canada, Thailand (1,818.44 Mil. Lit. – 2%), Argentina, and the rest of the world are in the beginning to produce bioethanol (RFA, 2021).

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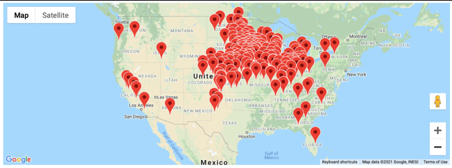

- The U.S. has the world's fastest growing and largest fuel bioethanol market.
- Presently, there are 210 and growing bioethanol plants in the U.S (RFA, 2021) which mostly using corn starch as feedstock (substrate).
- The 210 bioethanol plants located across 27 states in U.S. produced an amazing 16.1 billion gallons of fuel bioethanol.
- Total consumption increased to 16.2 billion gallons, 300 million gallons more than a year ago, driven mostly by record exports of over 1.6 billion gallons as global octane demand continues to grow (RFA, 2021).
- In recent years fuel ethanol demand has been stimulated by the phasing-out of MTBE as octane enhancer.
- A major concern with methyl tertiary butyl ether (MTBE) is water contamination and its health effects.

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- In 2012, bioethanol produced from corn in the U.S. was estimated to have cost between USD 0.9 and USD 1.1 per litre of gasoline equivalent (LGE) to produce.
- In contrast, Brazilian sugarcane bioethanol was estimated to have cost between USD 0.7/LGE and USD 0.9/LGE due to corn starch needs to be converted to sugar prior to fermentation.
- The cost of ethanol from other grains (e.g. wheat) was higher.
- This compares to average refinery wholesale prices in the United States, with monthly averages between USD 0.72/litre and USD 0.84/litre in 2012 for gasoline (petrol).
- Current price of bioethanol in the U.S. is USD 0.75/litre compare to USD 0.92/litre in November 22nd 2021 (Trading Economics, 2021).
- Since 1975, with the creation of the National Alcohol Program (Proalcool) by Brazilian Government has produced bioethanol from sugarcane; this alcohol is blended with petrol in proportions of up to 25%.
- Brazil has been for decades the world's largest producer and consumer of bioethanol, but was overtaken by the U.S. in 2006.

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- Currently, Brazil is the world's largest producer of sugarcane, with crops often yielding over 300 million tonnes of crushed cane per harvest season (Bajay, 2004).
- Brazil is now the world's second largest bioethanol producer in the world and is the largest exporter of bioethanol which were estimated at 1.8 billion liters, an increase of 11% in comparison to total exports in 2018 (1.62 billion liters).
- At the same time, Brazil also imports bioethanol for fuel use and originate almost totally from the U.S. (EIA, 2021).
- Today, there is no ethanol-free gasoline (petrol) on the Brazilian fuel market as all petrol is marketed with a 27% share of bioethanol (E27, also called gasohol).
- Present price of bioethanol in Brazil is USD 0.96/litre with an average value of USD 0.87 (GlobalPetrolPrice.com).
- The total number of bioethanol plants in 2021 is 365 units, includes 343 sugarcane-based ethanol plants, 7 corn-based ethanol plants, 12 flex plants producing ethanol from both sugarcane and/or corn, and 3 lignocellulosic bioethanol plants (USDABrazil.org.br, 2021).

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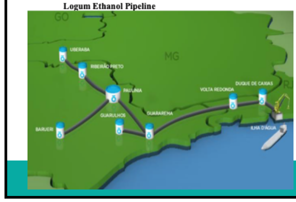
Logum Ethanol Pipeline



- The map beside shows the location of the bioethanol plants in Brazil.
- The total hydrated bioethanol production capacity for 2021 is reported at 58.5 billion liters per year.
- This figure reflects the authorized hydrated bioethanol production capacity of 243.842 million liters per day, as reported by ANP (National Agency of Petroleum, Natural Gas & Biofuels), and assumes an average of 240 crushing days.
- Brazil has one bioethanol pipeline, which is operated by Logum, which currently connects Brazil's principal ethanol-producing regions in the Center-South with major fuel consumer centers such as the metropolitan regions of Sao Paulo and Rio de Janeiro.
- The pipeline currently extends 1,400 km and has the capacity to move 4 billion liters of bioethanol per year.

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- The Alternative Energy Development Plan (AEDP 2018), approved in April 2019, set a goal that 30% of total energy consumption in Thailand will come from renewable energy sources by 2037.
- One of its initial steps is to replace the octane-enhancing additive MTBE in gasoline with bioethanol.
- Large-scale production of bioethanol has started with molasses but cassava was officially designated the prime raw material.
- Thailand is one of the largest producer of cassava with nearly 32 million of tonnes in 2018 (FOASTAT, 2020).
- As the domestic price of sugar is too high, it doesn't seem worth to produce bioethanol from sugarcane.
- In Thailand, the plan to make E20 gasohol as the primary petrol in the country has been postpone.
- It had been announced at the start of 2020 that the country would begin widespread adoption of the fuel – which is composed of 20% bioethanol blended with 80% unleaded gasoline 95 petrol – last year as a replacement for E10 (gasohol 91), but the appearance of the COVID-19 pandemic changed the course of things.

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- According to the country's Oil Fuel Fund Administration Office (OFFO), which subsidises gasohol and biodiesel, prices of ethanol and palm oil-made methyl ester have increased for many years, exceeding even those for pure gasoline and diesel, and pushing on with E20 adoption would create a financial burden for the Oil Fuel Fund.
- It was reported that there were 3.3 million E20-compatible cars out of a total of 5 million cars registered in Thailand, and around 6.5 million litres of E20 were being consumed a day.
- Raw materials for domestic bioethanol production in Thailand are sugarcane, molasses and cassava.
- Due to shortages of these feedstocks, Thailand will be forced to temporarily lower biofuel use targets or price surges when weather-related feedstock shortages occur, and the country's lack of ethanol imports will stop it from meeting higher targets of bioethanol use.
- The decreased rate of ethanol production and use is due to the delay in the cessation of Octane 91 E10 sales earlier scheduled on January 1, 2018 but now rescheduled to January 2022.
- Inspite of the delay, the ethanol-blend levels in the country have reached 13.5% in this year as a result of strong E20 sales (http://www.ethanolproducer.com/articles/16741/report-thailand- could-benefit-from-ethanol-imports).

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- The postponed was due to concerns about limited supplies of molasses, which is the primary feedstock for ethanol production. Molasses-based ethanol production is expected to further decline in 2021.
- Supplies of molasses have been tight due to reduced sugarcane production for the second consecutive year.
- Bioethanol demand is primarily expected to be fulfilled by cassava-based.
- The bioethanol consumption target in the AEDP 2018 is 2,700 million liters in 2037, down 34% from the 2015 target of 4,100 million liters.
- The lowered target is in anticipation of limited supplies of molasses and cassava.
- Additionally, long-term demand growth for gasoline and gasohol is expected to slow down in the long run due to increased availability of passenger and commercial Electric Vehicles (EVs) and the operation of double-track railways, which are under construction.
- The government expects ethanol consumption will decelerate in 2025 when the number of EVs on the road reaches the target in the AEDP 2018.
- The increase in the number of EVs on the road is also partly aligned with the goal to reduce GHS emission by 2035.

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3.0 BIOETHANOL FEEDSTOCKS AND PRETREATMENT PROCESSES

$$\underset{\text{sugar (e.g.: glucose)}}{C_6H_{12}O_6} \xrightarrow{\textit{S. cerevisae}} \underset{\text{bioethanol}}{2\ C_2H_5OH} + \underset{\text{carbon dioxide}}{2\ CO_2}$$

- Refer to the stoichiometric above, in general bioethanol can be produced through fermentation reaction from any feedstocks that has the typical formula of $(CH_2O)_n$.
- These feedstocks can be divided in 3 main groups:
  - a) sugar-based (sugarcane, sugar beet & sweet sorghum
  - b) starch-based (wheat, corn & cassava)
  - c) lignocellulosic biomass (straw, grasses, wood, stover, agricultural waste residues etc.)
- **First-generation** bioethanol are produced from sugar and starch feedstock; **second-generation** bioethanol from lignocellulosic biomass; **third-generation** biomass from micro/macroalgea biomass; **fourth-generation** from genetically modified cynobacteria through 'photofermentation' (direct conversion of light and CO_2 into ethanol) (Silva and Bertucco, 2016).

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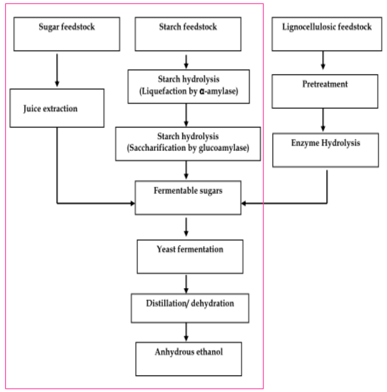

Bioethanol production by fermentation process of sugar, starch and lignocellulosic feedstock.

- Bioethanol production begins with obtaining solution that contains fermentable sugars as substrate.
- The feedstock selection totally depends on location and dominant local agricultural feedstock such as corn (USA), sugarcane (Brazil), wheat (France, England, Germany, and Spain), cassava (Thailand, Nigeria) and sorghum (India).
- Most current bioethanol production processes utilize more readily degradable biomass feedstock such as cereals (corn or grain) and sugarcane juice.
- However, the utilization of edible agricultural crops exclusively for bioethanol production conflict with food and feed production.

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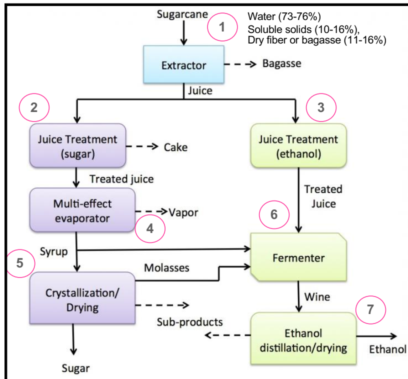

Process of sugarcane to produce sugar and bioethanol

https://www.e-education.psu.edu/egee439/node/647

- Stage 1 (Extraction) results in juice [also produces bagasse]. Juice is sent on to two possible stages: Stage 2 (eventually leading to sugar) or Stage 3 (eventually leading to ethanol).
- Stage 2 produces Treated Juice [also produces cake].
- Stage 3 produces Treated Juice. Treated Juice from Stage 3 is sent on to Stage 6 (Fermentor), while Treated Juice from Stage 2 is sent on to Stage 4 (Multi Effect Evaporator).
- The result of Stage 4 (Multi Effect Evaporator) is syrup [this stage also produces vapor]. This syrup is sent on in several possible directions.
  - Option 1. The syrup finishes at Stage 5 (Crystallization, Drying), where the syrup becomes sugar [or sub-products].
  - Option 2. The syrup passes through Stage 5 (Crystallization, Drying), where the syrup becomes molasses and then goes on to Stage 6 (Fermentor).
  - Option 3. The syrup goes directly to Stage 6 (Fermentor).
  - The result of Stage 6 (Fermentor) is wine. The wine is sent on to Stage 7.
- The result of Stage 7 (Ethanol Distillation) is ethanol [this stage also produces sub-products].

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- Sugarcane, sugar beet and sweet sorghum are the main sucrose-containing feedstocks for bioethanol production with feedstock yields of 62 - 74 tonnes·ha^{-1} (Almodares & Hadi, 2009), 54 - 111 tonnes·ha^{-1} (Vohra et al., 2014) and 50 - 62 tonnes·ha^{-1} (Almodares & Hadi, 2009), respectively and are mostly exploited in Brazil, France, Germany and India.
- Sugarcane molasses from sugarcane processing, aqueous juice expelled from sugar beets and sweet sorghum stalks were employed as raw material in bioethanol production.
- The proximate composition of sugar-based and starch-based feedstocks are as follow:

Sugarcane molasses [8]		Sugar beet juice [9]		Sweet sorghum stalks [10]			Corn grain [11]	Wheat grain [12]	Cassava [13]
Component	% w	Component	% w	Component	% w	Component	% w	% w	% w
Water	18.9	Water	65.6	Cellulose	8.7	Starch	72.0	53.0 - 57.0	77.0 - 94.0
Sucrose	31.8	Solids	17.3	Hemicellulose	6.3	Fiber	9.5	9.9 - 11.8	1.5 - 3.7
Invert sugar	15.4	Sucrose	16.5	Lignin	0.6	Sugars	2.6	0.0	0.0
Ash	13.8	Sugars	0.2	Sucrose	67.4	Protein	9.5	12.5 - 15.1	1.7 - 3.8
Others	20.1	Impurities	0.3	Glucose	3.7	Oil	4.3	2.1 - 2.6	0.2 - 1.4
				Ash	0.2	Minerals/Ash	1.4	0.0	1.8 - 2.5
				Others	13.1	Water	0.0	12.0	59.0 - 70.0
						Others	0.7	4.9 - 5.8	0.0

Proximate composition of starch-containing and sucrose-containing feedstock (Reproduce by Mukhtham et al., 2016).

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- Sugarcane molasses is composed of sucrose (31%) and inverted sugar (15%). Therefore, sucrose concentration in sugarcane molasses must be diluted (to 14% - 18%) before fermentation to facilitate the optimum growth of fermenting microorganism (substrate inhibition).
- The juice extracted from sugar beet is composed of 16.5% sucrose, whereas in sweet sorghum, stalks are the main store of sugar and are mechanically pressed to recover a sugar juice of 12% - 22% concentration which can be directly fermented anaerobically by *Saccharomyces cerevisiae* (yeast) to produce bioethanol.
- Although bioethanol production using sugar-based feedstock has been well reported, research is still ongoing, including the testing of different yeast species available in the market and also newly isolated species to achieve high ethanol yields and to reduce the formation of foam and glycerol during fermentation.
- Foaming and glycerol formation are the major parameters which can have a significant impact on ethanol production costs.
- A summary of the latest research reports on ethanol production from sucrose-containing feedstocks together with feedstock availability is presented in the next page.

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Feedstock	Yield, tonnes ha^{-1}	Sugar content, % w/w	Fermentation organism/conditions	Ethanol
Sugarcane molasses	62 - 74 (Sugarcane) [6]	31% sucrose and 15% invert sugars	New Aule Alcohol yeast and New Aule Baker's yeast; fermentation at room temperature, pH 4.3 for 72 h, inoculum 1% w/v	Alcohol yeast-74.8 g·L^{-1}, $Y_{p/s}$ 0.4 g·g^{-1} and Baker's yeast-102.9 g·L^{-1}, $Y_{p/s}$ 0.7 g·g^{-1} from 300 g·L^{-1} sugar concentration
Sugarcane molasses			*Saccharomyces species* isolated from molasses; fermentation at 30˚C for 144 h, inoculum 0.5 g·L^{-1}	128.7 g·L^{-1}, $Y_{p/s}$ 0.6 g·g^{-1} from 250 g·L^{-1} sugar concentration
Sugar beet molasses and thick juice	54 - 111 [7]	Total sugars in Sugar beet molasses: 53.0% and in thick juice: 60.0%	Immobilized yeast; fermentation at 30˚C, pH 5.5, 144 h, inoculum 1 g·L^{-1}	From molasses: $Y_{p/s}$ 0.5 g·g^{-1}, 96.8%, 83.2 g·L^{-1} and from thick juice: $Y_{p/s}$ 0.4 g·g^{-1}, 90.6%, 132.4 g·L^{-1} from 300 g·L^{-1} sugar concentration
Sugar beet raw, thin and thick juice and molasses		In raw juice:13.4% Thin juice:13.0% Thick juice: 58.3% Molasses: 50.1%	Commercial yeast strain; fermentation at 30˚C, 60 h, inoculum 3 g·L^{-1}	From raw juice: 0.08 v/v Thin juice: 0.08 v/v Thick juice: 0.08 v/v Molasses: 0.07 v/v from an initial sugar concentration of 130 g·kg^{-1} media
Sweet sorghum stalk juice	50 - 62 [6]	Sucrose 12% - 22%	Immobilized *S. cerevisiae* in bioreactor 5L; fermentation at 37˚C, pH 5, 12 h, inoculums 10^8 cells·mL^{-1}	33 mg·mL^{-1}, yield 98.0%
Sweet sorghum stalk juice			Immobilized *S. cerevisiae* in fluidized bed fermenter; fermentation at 32˚C, pH 4, 9 h, inoculum 10^8 cells·mL^{-1}	Ethanol content 6.2% v/v; yield 91.6%
Sweet sorghum juice			*S. cerevisiae;* fermentation at 30˚C, 48 h, inoculums 5^*10^8 cells·mL^{-1}	Ethanol 133.5 g·L^{-1}, 87.6% of the theoretical yield
Sweet sorghum juice			*S. cerevisiae* strain BY4741; fermentation at 30˚C, pH 5.2, 48 h, inoculum 5^*10^8 cells·mL^{-1}	Ethanol 115.2 g·L^{-1}, 87.1% of the theoretical ethanol yield, $Y_{p/s}$ 0.4 g·g^{-1}
Sweet sorghum juice from three varieties: GK-coba; Mn-4508; SS-301		GK-cba: 16.9%, Mn-4508: 17.4%, SS-301: 19.1%	*Zymomonas mobilis* and *S. cerevisiae* mixed culture (1:1); fermentation at 30˚C, 4 days, inoculum 5 mL of 48 h old liquid seed cultures	45.2 mL·L^{-1}; 1075.4 L·ha^{-1} 46.9 mL·L^{-1}; 1318.2 L·ha^{-1} 50.2 mL·L^{-1}; 1232.6 L·ha^{-1}

Reproduce by Muktham et al., 2016).

- A summary of the latest research reports on ethanol production from sucrose-containing feedstocks together with feedstock availability:
- The main points are:
  - 3 days fermentation at mild condition using New Aule Baker's yeast resulted in 102.9g/L bioethanol; $Y_{P/S}$ 0.7 g/g; 300g/L sugarcane molasses (Jayus et al., 2016).
  - 6 days fermentation at mild condition using yeast isolated from molasses resulted in 128.7g/L bioethanol; $Y_{P/S}$ 0.6 g/g; 250g/L sugarcane molasses (Muruaga et al., 2016)

Which is better???

$Y_{P/S}$ = Yield of product (g)/substrate (g)

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- What is the highest bioethanol that can be produced theoretically?
- Theoretical yield and percent yield of bioethanol: ?
- Based on stoichiometry and molar mass, we have:

$$\underset{\text{sugar (e.g.: glucose)}}{C_6H_{12}O_6} \xrightarrow{\textit{S. cerevisae}} \underset{\text{bioethanol}}{2\ C_2H_5OH} + \underset{\text{carbon dioxide}}{2\ CO_2}$$

$$300\text{ g Glucose} \times \frac{1\text{ mol Glucose}}{180\text{g Glucose}} \times \frac{2\text{ mol EtOH}}{1\text{ mol Glucose}} \times \frac{46\text{ g EtOH}}{1\text{ mol EtOH}} =$$

$$300\ \cancel{\text{g Glucose}} \times \frac{\cancel{1\text{ mol Glucose}}}{\cancel{180\text{ g Glucose}}} \times \frac{2\ \cancel{\text{mol EtOH}}}{\cancel{1\text{ mol Glucose}}} \times \frac{46\text{ g EtOH}}{1\ \cancel{\text{mol EtOH}}} =$$

= 153.3 g EtOH can be produced theoretically from 300g Glucose
= 102.9g / 153.3g x 100
= 67% yield of bioethanol produced.

- Theoretical yield of bioethanol: ? (153.4g)
- Percent yield: ? Actual/Theoretical Yield (67%)
- Glucose used & balance: ? $Y_{P/S}$ = 0.7 (147 g Glucose used to produce 102.9 g Ethanol)

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- Sugar beet molasses and thick juice are the other promising raw sources for ethanol production due to their high sugar content i.e. 53.0% and 60.0%, respectively (Muktham et al., 2016).
- Molasses represents an almost complete fermentation medium as it comprises sugars (sucrose, glucose, fructose), minerals, vitamins, fatty acids, organic acids etc.
- These sugars can be converted to ethanol directly but starches must first be hydrolysed to fermentable sugars.
- Hydrolysis of starch to fermentable sugars can be known as saccharification where complex carbohydrates starch molecules (polysaccharides) are converted into simpler sugars (monomers).
- Acid hydrolysis of starch has had widespread use in the past and is now largely replaced by enzymic processes, as it required the use of corrosion resistant materials.
- A combination of bacterial **α-amylase** and fungal **glucoamylase** are normally used for liquefaction and saccharification of starch.
- Starch which is a polymer of glucose will be broken into glucose monomers by the action of **α-amylase** and **glucoamylase** enzymes, where simple sugar monomers can be fermented to produce bioethanol.

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- Corn, wheat and cassava are the most employed starch-based feedstocks in bioethanol production in North America, Europe and tropical countries, respectively.
- Conventionally, corn grain is converted to bioethanol by two methods; wet milling and dry milling.
- In wet milling corn grain is soaked in water to fractionate the grain into starch, fiber and germ involving separate processing of each fractionated component.
- Dry milling involves processing of whole grain and the residual components are separated at the end of the process.
- Wheat is another main cereal feedstock for grain distilleries and bioethanol production and it replaced barley 30 years ago.
- Dry milling of wheat to separate bran from grain improves the starch content in flour resulting in a high ethanol titer.
- Cassava is a promising feedstock for bioethanol production due to the high starch yield per hectare and availability of raw material all year round (36.3 tonnes.ha^{-1}annum^{-1}) (Wang, 2002).

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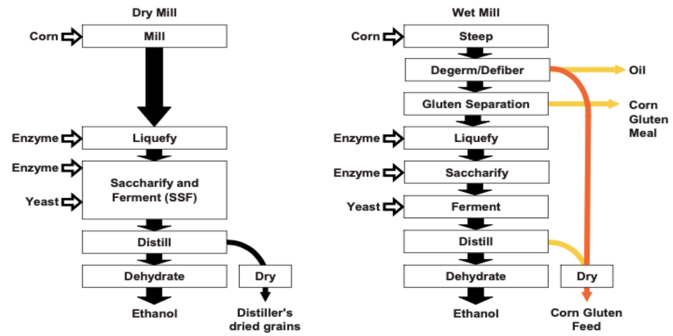

Dry and wet milling corn processes for bioethanol (Abbas, 2010)

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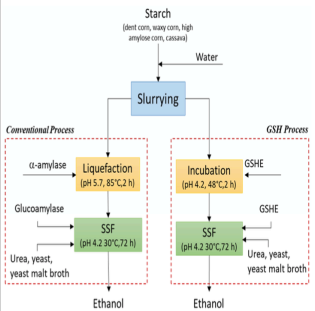

Conventional dry grind and granular starch hydrolysis (GSH) process for bioethanol production (Sarocha et al., 2018)

- Sarocha et al. (2018) compared the bioethanol production of cassava starch with corn starch using a conventional and a granular starch hydrolyzing process (GSH).
- Their experimental work combines liquefaction and simultaneous saccharification and fermentation (SSF).
- The final bioethanol concentration for dent corn starch (133.2 g/L) was the highest among the starch.
- For the cassava starch, the ethanol concentration (127.9 g/L) achieved with GSH process was 2.8% higher than that in the conventional process.
- However, statistically there was **no significant differences** in the final ethanol concentrations of the 3 starches—dent corn, waxy corn and cassava starch, which indicated the suitability of cassava starch to be used as feedstock (substrate) in Asian countries (cost effective).

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- Overall, the 1st generation bioethanol production from food crops have several limitations including the fact that it has a direct impact on food production in terms of food price, quality, and soil usage, for crop growth while providing only limited greenhouse gas emission reduction benefits.
- Currently there is much focus on advancing a cellulosic bioethanol concept (2nd generation) that utilizes lignocellulosic biomass.
- 2nd generation bioethanol produced from lignocellulosic biomass, non-food crops, industrial and municipal wastes results in greater greenhouse gas reductions and does not compete for agricultural land with food crops.
- The sustainability of substrate (continuous supply) to be used as feedstock has been the critical concern.
- Lignocellulosic biomass having the advantages and does not need to keep up with the commodity crops since it is a non-edible part of plant and can be considered as wastes.
- In economic point of view, compared to other feedstocks like starch, sugarcane and soy bean, lignocellulosic biomass is readily available and can be obtained at lower cost (Huang et al., 2011).

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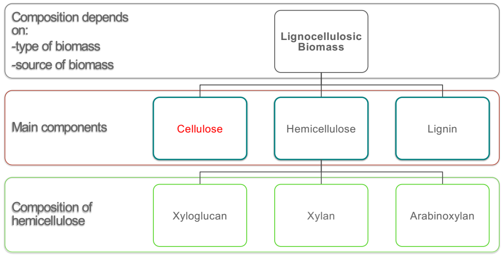

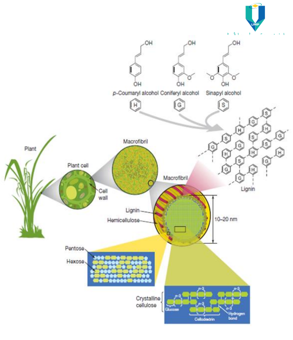

Structure of lignocellulosic biomass (Rubin, 2008)

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- As illustrated previously, lignocellulosic biomass having a complex bio-matrix with a cell wall made up of microfibril with a bunch of macrofibril.
- The components in cell wall or macrofibril can be categorized into two components with polysaccharides as a major part while another is a small portion of protein (Höfte & Voxeur, 2017).
- The polysaccharides in macrofibril comprised mainly from three different polymers; cellulose, hemicellulose and lignin, that organized to be a mediated structural stability in plant cell wall (Agbor et al., 2011; Rubin, 2008).
- These polymers are linked with each other and formed varying relative compositions depending on the type and source of biomass (Carere et al., 2008).
- Furthermore, the geographical location and climates variation of where the plants are grown up also be an important role towards the distribution of these compositions inside lignocellulosic biomass (Han & Rowell, 1997; Komuraiah et al., 2014; Michelin & Teixeira, 2016).

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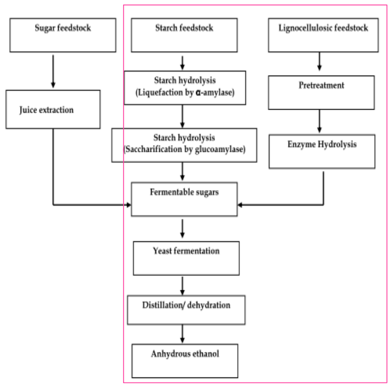

Bioethanol production by fermentation process of sugar, starch and lignocellulosic feedstock.

- Bioethanol production from lignocellulosic biomass feedstock typically comprises the following steps (Muktham et al., 2016):
  - Pre-treatment: process where the structural carbohydrates that compose the biomass are made more accessible for the subsequent steps;
  - Enzymatic hydrolysis: breakdown of the polymeric carbohydrates into simple sugars that can be fermented by the microorganisms into ethanol;
  - Fermentation: conversion of the carbohydrates into ethanol by the selected microorganism or culture;
  - Downstream processing: recovery of the ethanol from the fermentation broth (typically by distillation) and management of the remaining streams.

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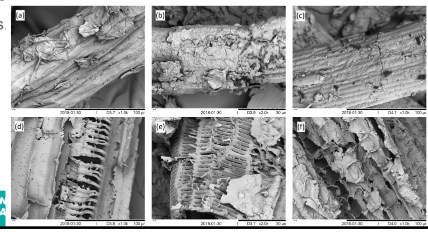
- The economic feasibility of biofuel production from lignocellulosic feedstock largely depends on:
  - (i) the type of biomass
  - (ii) the pretreatment process before fermentation.
- Availability, cost, transportation to the processing facility and physical state of the biomass are major factors affecting the selection of feedstock for bioethanol production.
- Agricultural residues and pulp/bagasse generated from 1st generation bioethanol process represent a promising feedstock for 2nd generation bioethanol production.
- The need for a pre-treatment step is the major distinction between a 1st and a 2nd generation bioethanol process.



SEM images on:
(a) non-treated OPFB,
(b) non-treated OPEFB,
(c) non-treated RS,
(d) pretreated OPFB,
(e) pretreated OPEFB and
(f) pretreated RS

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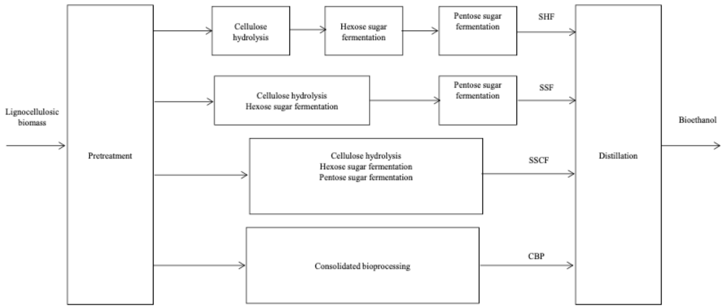

Process steps in lignocellulosic ethanol production reproduced from (Chiaramonti et al., 2012)
Separate hydrolysis and fermentation steps (SHF)
Simultaneous saccharification and co-fermentation (SSCF)
Consolidated bioprocessing step (CBP)

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- Existing bioethanol production processes have:
  - (i) separate hydrolysis and fermentation steps (SHF) (Dahnum et al., 2015);
  - (ii) simultaneous saccharification and fermentation (SSF) (Liu et al., 2015) refers to saccharification and fermentation of hexose sugars taking place within the same bioreactor;
  - (iii) simultaneous saccharification and co-fermentation (SSCF) refers to the saccharification and co-fermentation of both pentose and hexose sugars in a single step and
  - (iv) consolidated bioprocessing step (CBP) (Chiaramonti et al., 2012)
- In CBP, a single organism is used to produce the enzymes required and to perform both cellulose hydrolysis and fermentation (Horisawa et a., 2015).
- CBP is considered potentially the most cost-effective process as the processes, namely enzyme production, hydrolysis and fermentation are taking place within the same bioreactor making the capital cost lower (Olson et al., 2012).
- Hydrolysis of lignocellulosic substrates results in the formation of both hexose and pentose sugars from cellulose and hemicellulose, respectively.

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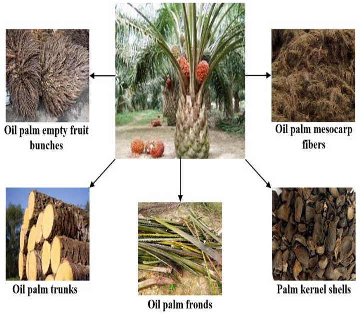
Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Author
Barley straw	46.0	23.0	15.0	Zhu et al. (2015)
Coconut fiber	31.6	25.5	35.1	Nascimento et al. (2016)
Corn cob	35.7	32.8	15.4	Ma et al. (2018)
Corn stover	37.1	20.9	13.5	Qing et al. (2017)
Kenaf fiber	60.5	19.9	15.8	Wang et al. (2016)
Oil palm empty fruit bunch	36.1	22.4	26.4	Tang et al. (2018)
Oil palm frond	41.9	33.6	20.7	Wee Lai et al. (2016)
Rice husk	36.8	19.7	35.3	Wu et al. (2018)
Rice straw	36.4	20.3	26.6	Harun & Geok (2016)
Sugarcane bagasse	43.4	25.1	14.7	Brienzo et al. (2015)
Wheat straw	37.4	27.9	18.4	Liu et al. (2015)

Compositions of various lignocellulosic biomass



Types of oil palm biomass

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- Pretreatment has a significant effect on the overall process which makes the hydrolysis easier and produces higher amount of fermentable sugars.
- Methods that are currently used for pretreatments are physical, chemical, biological and physicochemical.
- Physical pretreatment uses mechanical milling to ground the substrate.
- The common chemical pretreatment includes ozonolysis, acid hydrolysis, alkaline hydrolysis and organosoly based process.
- Different fungal species are involved in biological pretreatment while physicochemical pretreatment includes ammonia fiber explosion (AFEX) and steam.
- Dehydration of hexose and pentoses during pretreatment release furan compounds like 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde.
- These furan derivatives induce the inhibition of cell growth and reduce ethanol productivity.
- Yeasts fermentation can be inhibited by the weak acid stress induced from lignocellulosic materials.

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Pretreatment	Method	Operating conditions	Advantages	Limitations	Author
Physical	Pyrolysis, freezing, extrusion, microwave, milling	Room temperature or very high temperature. Energy input less than 30 kW per ton biomass.	Reduces cellulose crystallinity.	Power consumption higher than inherent biomass energy. Too expensive to be used in a full-scale process.	Kumari & Singh (2018)
Thermal	Steam	Temperature: 160 – 260 °C Pressure: 0.69 – 4.83 MPa Time: 5 – 15 min	Autohydrolysis of hemicelluloses and lignin transformation. Cost effective for hardwoods and agricultural.	Incomplete destruction of lignin-carbohydrate matrix. Generation of inhibitors. Destruct some portion of xylan. Less effectives for softwood.	Sun & Cheng (2002)
Chemical	Acid	Operating at lower or higher temperature depending on product.	Hydrolyzes hemicelluloses to xylose and other sugars. Alters lignin structure.	Toxics formation. Equipment corrosion. Hazardous. Expensive due to the need of reactors that resistant to corrosion.	Jung & Kim (2015)
	Alkali	Low operating temperature. Long pretreatment time. High base concentration.	Remove hemicellulose and lignin. Increase accessible surface area.	Left residuals salt in biomass.	Mosier et al. (2005)
Biological	Microbial degradation	Several fungi (brown, white, soft rot)	Degrade lignin and hemicellulose. Low energy requirements.	Slow hydrolysis rate.	Galbe & Zacchi (2007); Lee et al. (2008)

Summary of pretreatment methods for lignocellulosic biomass

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- The most commonly used pretreatment method is steam explosion.
- This is contributed by the attractive features of steam explosion which has less environmental impact, low capital investment, high energy efficiency, less hazardous process chemicals and conditions and complete sugar recovery [83].
- After the delignification of materials through the performance of **pretreatment** process, commonly bioconversion of lignocellulosic biomass will be pursued with **hydrolysis** step to achieve the desired product.
- **Hydrolysis is the process to convert biopolymer of biomass into fermentable sugars**.
- This can be accomplished either by the traditional way of using acid as hydrolysis catalyst, or the latest route is through the digestibility of enzyme (Verardi et al., 2012).
- Elements that will influence the hydrolysis of lignocellulosic materials including the porosity or accessible surface area of lignocellulosic biomass, the crystallinity of biomass and content of the materials depending upon the desired product (Kumar et al., 2009).
- Acidic hydrolysis can be divided into two types namely dilute and concentrated.

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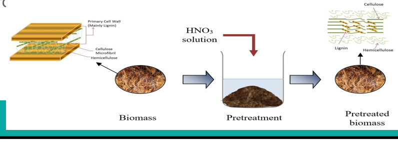
- Dilute acid hydrolysis is performed at higher temperature using low acid concentration while concentrated acid hydrolysis is carried out at lower temperature using high acid concentration.
- Dilute acid hydrolysis is the most commonly used process compared to concentrated acid hydrolysis.
- However, it may generates large amount of inhibitors like 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde.
- Acid hydrolysis of lignocellulosic biomass is conducted in two-stage process as the pentose sugars degrade more rapidly compared to hexose sugars.
- Hemicellulose is hydrolysed in the first stage using dilute acid while cellulose is hydrolysed in the second stage using much concentrated acid.
- Concentrated acid process generates high sugar recovery (90%) in shorter period of time (Joshi et al., 2011).
- The disadvantages of acid hydrolysis are the difficulty of performing acid recovery and recycling process which increases the production cost.



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- Enzymatic hydrolysis requires enzymes to hydrolyse the feedstocks into fermentable sugars.
- Three types of enzymes that are commonly used for cellulose breakdown such as endo-β—1,4-glucanases, cellobiohydrolases, and β-glucosidases.
- The activity of cellulase enzyme is influenced by their concentration and source of the enzyme.
- Cellulose will be degraded into reducing sugars under mild reaction conditions (pH: 4.8–5.0, T: 45–50°C).
- Moreover, it does not cause corrosion problem in the reactors which can result in high sugar yields.
- The efficiency of enzymatic hydrolysis is influenced by optimized conditions such temperature, time, pH, enzyme loading and substrate concentration (Chandel et al., 2007)
- Enzymatic saccharification of cellulose can be enhanced by using surfactants such as polyethylene glycol (PEG) or Tween 20, which function to block lignin and reduce the adsorption of cellulase on lignin (Joshi et al., 2011).
- Used to be the limitation of using enzymes in hydrolysis is because they are too expensive for the economical production of ethanol from biomass, now there are more cheaper enzymes from Novozyme in the market.
- Enzymatic hydrolysis is the preferred saccharification method because of its higher yields, higher selectivity, lower energy cost and milder operating condition than chemical processes (Yang et al., 2011).

YouTube https://www.youtube.com/watch?v=T4Cs2LQcWhU

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4.0 BIOETHANOL FERMENTATION ASPECTS (UPSTREAM)

- As mentioned earlier, bioethanol can be produced from various types of feedstocks such as sucrose, starch, and lignocellulosic biomass through fermentation process by microorganism.
- Compared to other types of microorganism, yeasts especially *Saccharomyces cerevisiae* is the common microbes employed in ethanol production due to its **high ethanol productivity, high ethanol tolerance and ability of fermenting wide range of sugars**.
- However, there are some challenges in yeast fermentation which inhibit ethanol production such as high temperature, high ethanol concentration and the ability to ferment pentose sugars.
- Various types of yeast strains have been used in fermentation for ethanol production including **hybrid, recombinant and wild-type yeasts.**
- This section will discuss on the bioethanol fermentation aspects including preparation of inoculum, bioreactor, and fermentation processing parameters.

$$\underset{\text{sugar (e.g.: glucose)}}{C_6H_{12}O_6} \xrightarrow{S.\ cerevisae} \underset{\text{bioethanol}}{2\ C_2H_5OH} + \underset{\text{carbon dioxide}}{2\ CO_2}$$

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- The common processes involves in bioethanol production are feedstock pretreatment & hydrolysis, fermentation and separation and purification.
- Generally, there are three major steps in bioethanol production:
  (1) obtaining solution that contains fermentable sugars;
  (2) converting sugars to ethanol by fermentation; and
  (3) ethanol separation and purification.
- Feedstocks are usually pretreated in order to reduce its size, and facilitate subsequent processes.
- Then, the hemicellulose and cellulose will be hydrolysed to fermentable sugars pentose and hexose, respectively.
- Yeasts are given the responsibility to ferment these sugars into ethanol.
- Separation technologies are used to recover bioethanol before it can be used as fuel.

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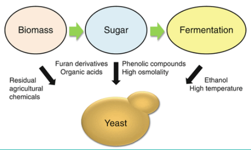
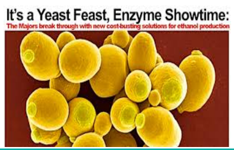
- Yeasts can directly ferment simple sugars into ethanol while other type of feedstocks must be converted to fermentable sugars before it can be fermented to ethanol.
- Production of bioethanol during fermentation depends on several factors such as temperature, sugar concentration, pH, fermentation time, agitation rate, and inoculum size.
- The efficiency and productivity of bioethanol can be enhanced by immobilizing the yeast cells.
- Yeasts are used in industrial plants due to valuable properties in bioethanol yield (> 90.0% theoretical yield), ethanol tolerance (> 40.0 g/L), bioethanol productivity (> 1.0 g/L/h), growth in simple, inexpensive media and undiluted fermentation broth with resistance to inhibitors and retard contaminants from growth condition (Dien et al., 2003).





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- Since thousands of years ago, yeasts such as *S. cerevisiae* have been used in alcohol production especially in the brewery and wine industries.
- Baker's yeast was traditionally used as a starter culture in bioethanol production due to its low cost and easy availability.
- It keeps the distillation cost low as it gives a high ethanol yield, a high productivity and can withstand high ethanol concentration (Kasavi et al., 2012).
- Certain yeast strains such as *Pichia stipitis* (NRRL-Y-7124), *S. cerevisiae* (RL-11) and *Kluyveromyces fagilis* (Kf1) were reported as good ethanol producers from different types of sugars (Mussato et al., 2012).
- *S. cerevisiae* is the most commonly employed yeast in industrial bioethanol production as it tolerates a wide range of pH thus making the process less susceptible to infection (Lin et al, 2012).
- Flocculent yeasts were also used during biological fermentation for ethanol production as it facilitates downstream processing, allows operation at high cell density and gives higher overall productivity (Basso et al., 2008; Domingues et al., 2000).

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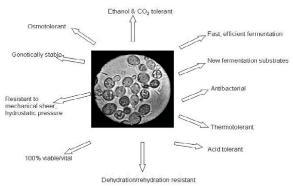
- Flocculent yeasts reduces the cost of cells recovery as it separate easily from the fermentation medium without centrifugation (Choi et al., 2010).
- Stressful conditions like an increase in ethanol concentration(over 20%), rise in temperature (35–45 °C), osmotic stress and bacterial contamination are the reasons why the yeast cannot survive during the fermentation (Basso et al., 2008).
- Increase in ethanol concentration during fermentation can cause inhibition to microorganism growth and viability (Alexandre and Charpentier, 1998).
- Inability of *S. cerevisiae* to grow in media containing high level of alcohol leads to the inhibition of ethanol production (Fiedurek, 2011).
- The other problems in bioethanol fermentation by yeast are the ability to ferment pentose sugars.
- *S. cerevisiae* can only ferment hexoses but not pentoses as only some yeasts from genera *Pichia, Candida, Schizosaccharomyces* and *Pachysolen* are capable of fermenting pentoses to ethanol (Mussato et al., 2012).

- The problems of pentose fermentation is solved by using hybrid, genetically engineered or co-culture of two yeast strains.
- Hybrid yeast strains are used simultaneously to ferment pentose and hexose sugars to ethanol.
- Whereas genetic engineering use recombinant DNA technology to up-regulate the stress tolerance genes in order to overcome the inhibitory situations (Dogan et al., 2014).
- Genetically engineered *S. cerevisiae* and co-culture of two strains have been developed to produce bioethanol from xylose with high yield.
- The engineered yeast strains can convert cellulose to ethanol more rapidly compared to unmodified yeast strains.
- Co-culture process simultaneously culture and grow two different yeasts in the same bioreactor (Tanimura et al., 2012).
- Co-culture shows better ethanol production as compared to its pure culture as in co-culture, pentose utilizing yeasts like *Pichia fermentans* and *Pichia stipitis* are combined together with *S. cerevisiae* so that hexose and pentose sugars can be efficiently utilized.

Yeast strain	Type of strain	Feedstock	Sugar concentration (g/L)	Fermentation condition	Ethanol concentration (g/L)	Ethanol productivity (g/L/h)
S. cerevisiae RL-11	Laboratory	Spent coffee grounds	195.0	30 °C, 200 rpm, 48 h	11.7	0.49
S. cerevisiae MTCC 173	Laboratory	Sorghum stover	200.0	30 °C, 120 rpm, 96 h	68.0	0.94
S. stipitis CBS 6054	Laboratory	Giant reed	33.4	30 °C, 150 rpm, 96 h	8.2	0.17
S. cerevisiae KL17	Wild-type	Galactose and glucose	500.0	30 °C, 200 rpm, 28 h	96.9	3.46
S. pombe CHFY0201	Wild-type	Cassava starch	95.0	32 °C, 120 rpm, 66 h	72.1	1.16
S. cerevisiae CHY1011	Wild-type	Cassava starch	195.0	32 °C, 120 rpm, 66 h	89.1	1.35
S. cerevisiae ZU-10	Recombinant	Corn stover	99.0	30 °C, 180 rpm, 72 h	41.2	0.57
S. cerevisiae RPRT90	Mutated hybrid	*Ipomea carnea*	72.1	30 °C, 150 rpm, 28 h	29.0	1.03
S. cerevisiae CHFY0321(protoplast fusant)	Hybrid	Cassava starch	195.0	32 °C, 120 rpm, 65 h	89.8	1.38

Yeast strains used in bioethanol production (Reproduce by Mohd Azhar et al., 2017).

- The highest ethanol concentration of 96.9g/L with a productivity of 3.46g/L/h was produced by the wild-type yeast strain used, *S. cerevisiae* KL17 which is capable of utilizing both glucose and galactose simultaneously.
- It shows that wild-type yeasts (control strain that has not been genetically modified) has high potential in fermenting sugars to ethanol.



Desired attributes for bioethanol yeasts

- In order to grow and ferment, yeast cells require a range of essential nutrients.
- These can be categorized as:
  - a) macronutrients (sources of carbon, nitrogen, oxygen, sulphur, phosphorus, potassium, and magnesium) required at the millimolar level in growth media;
  - b) micronutrients (sources of trace elements such as Ca, Cu, Fe, Mn, Zn) required at the micromolar level.
- Most yeasts grow quite well in simple nutritional media, which supplies carbon and nitrogen-backbone compounds together with inorganic ions and a few growth factors.
- The latter are organic compounds required in very low concentrations for specific catalytic or structural roles in yeast, but are not used as energy sources.
- Growth factors for yeast include vitamins, which serve vital functions as components of coenzymes; purines and pyrimidines; nucleosides and nucleotides; amino acids; fatty acids; sterols; and other miscellaneous compounds (e.g., polyamines and choline).

- There are three processes that are commonly used in bioethanol production which are separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF).
- In SHF, hydrolysis of lignocellulosic materials is separated from ethanol fermentation.
- The separation of enzymatic hydrolysis and fermentation allows enzyme to be operated at high temperature for better performance while fermentation organisms can be operated at moderate temperature for optimizing sugar utilization.
- SSF and SSCF have a short overall process as the enzymatic hydrolysis and fermentation process occur simultaneously to keep the concentration of glucose low.
- For SSF, the fermentation of glucose is separated from pentoses while SSCF ferment glucose and pentoses in the same reactor (Canilha et al., 2012).
- Both SSF and SSCF are preferred over SHF because the operation can be performed in the same tank.
- The benefits of both processes are **lower cost, higher ethanol yield and shorter processing time** (Chandel et al., 2012).

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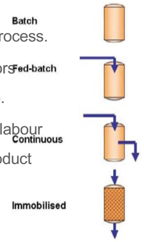
- Fermentation of bioethanol can be carried out in batch, fed-batch, repeated batch or continuous mode.
- In batch process, substrate is provided at the beginning of the process without addition or removal of the medium (Hadiyanto et al., 2013).
- It is known as the simplest system of bioreactor with multi-vessel, flexible and easy control process.
- The fermentation process is carried out in a closed-loop system with high sugars and inhibitors concentration at the beginning and ends with high product concentration (Thatoi et al., 2014).
- There are several benefits of batch system including complete sterilization, does not require labour skills, easy to manage the feedstocks, can be can be control easily and fexible to various product specifications (Ivanova et al., 2011).
- However, the productivity is low and need intensive and high labour costs.
- The presence of high sugar concentration in the fermentation medium may lead to substrate inhibition and results in the inhibition of cell growth and ethanol production (Cheng et al., 2009).
- Repeated-batch fermentation can be performed by replacing free cells with the immobilized cells.



Fermentation systems for bioethanol production

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- Fed-batch fermentation is a combination of batch and continuous mode which involves the addition of substrate into the fermenter without removing the medium.
- It has been used to overcome the problem of substrate inhibition in batch operation.
- Volume of culture in fed-batch processes can vary widely but it must be fed properly at certain rate with the right component composition.
- Productivity of fed-batch fermentation can be increased by maintaining substrate at low concentration which allows the conversion of sufficient amount of fermentable sugars to ethanol (Jain and Chaurasia, 2014).
- This process has higher productivity, higher dissolved oxygen in medium, shorter fermentation time and lower toxic effect of the medium components compared to other types of fermentation (Cheng et al., 2009).
- However, ethanol productivity in fed-batch is limited by feed rate and cell mass concentration (Choi et al., 2009).
- Fed-batch operation has been applied successfully in non-uniform SSF system by continuously adding a pretreated substrate in order to achieve relatively high sugar and ethanol concentration (Kang et al., 2014).

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- Continuous operation is carried out by constantly adding substrates, culture medium and nutrients into a bioreactor containing active microorganisms.
- Culture volume in continuous operation must be constant and the fermentation products are taken continuously from the media.
- Various type of products can be obtained from the top of the bioreactor such as bioethanol, cells and residual sugar (Ivanova et al., 2011).
- The advantages of continuous system over batch and fed-batch system are higher productivity, smaller bioreactor volumes and less investment and operational costs (Jain and Chaurasia, 2014).
- At high dilution rate, ethanol productivity is increased while ethanol yield is decreased due to incompletely substrate consumption by yeasts (Sanchez and Cardano, 2008).
- However, the possibility for contamination to occur is higher than other types of fermentation (Chandel et al. 2007).
- Moreover, the ability of yeasts to produce bioethanol in continuous process are reduced due to long cultivation time.

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Fermentation system (brief description)	Advantages	Disadvantages
Batch (microbial inoculum introduced into fermentation medium and left until complete)	Large capacity. Simple, robust, traditional (eg. brewing). Ease of sterilisation and cleaning. Complete substrate conversion.	μmax (short). Unbalanced, asynchronous. Low productivity Low cell densities. Labour intensive.
Fed-batch (nutrient fed incrementally, or batch-wise, to a growing yeast culture)	Traditional (baker's yeast) and modern (therapeutic proteins). Extends exponential phase (high cell densities). Complete substrate conversion	Low μ. Unbalanced (growth rate). Labour intensive.
Continuous (nutrient fed into a growing yeast culture at a rate equal to removal of culture broth)	Steady-state system. Growth rate controlled by dilution rate [D=μ]. High productivity. Nutrient balanced (chemostat). Low labour costs and good utilisation of thereactor. Valuable research tool (eg. adaptive evolution).	Costly interruptions due to contamination and mutation of productions yeast strains.
Immobilised (cells entrapped in a polymeric matrix or immobilised on the surface of an inert support material)	High yeast concentration 10^8 - 10^9 cells/ml. Cheap support materials (eg. wood chips). Continuous operation	Un-tested on a large scale for bioethanol production.

Advantages and disadvantages of different fermentation systems for bioethanol

- Immobilized cell technology is commonly applied in fermentation process.
- The benefits of immobilized cells over free cells include higher cell density per volume of reactor, easier separation from the reaction medium, higher substrate conversion, less inhibition by products, shorter reaction time and control of cell replication (Duarte et al., 2013).
- Immobilization should be performed under mild condition to maintain the activity of the cells (Calinescu et al. 2012).
- The commonly employed method for yeast immobilization is adsorption because the cells are not affected and yeast can be added or washed out from the fermentation medium (Bai et al., 2008).

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- There are several factors (6) which influence the production of bioethanol including i) temperature; ii) sugar concentration; iii) pH; iv) fermentation time; v) agitation rate; and vi) inoculum size (Zabed et al., 2014).
- The growth rate of the microorganisms is directly affected by the temperature (Charoenchai et al., 1998).
- High temperature which is unfavorable for cells growth becomes a stress factor for microorganisms (Marelnecot et al., 1998).
- The ideal temperature range for fermentation is between 20 and 35 °C.
- Free cells of *S. cerevisiae* have an optimum temperature near 30 °C whereas immobilized cells have slightly higher optimum temperature due to its ability to transfer heat from particle surface to inside the cells (Liu and Shen, 2008).
- Moreover, enzymes which regulate microbial activity and fermentation process are sensitive to high temperature which can denature its tertiary structure and inactivates the enzymes (Phisalaphong et al., 2006).
- Thus, temperature is carefully regulated throughout the fermentation process.
- Generally, the maximum rate of ethanol production is achieved when using sugars at the concentration of 150 g/L.
- The initial sugar concentration also has been considered as an important factor in ethanol production.

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- High ethanol productivity and yield in batch fermentation can be obtained by using higher initial sugar concentration.
- However, it needs longer fermentation time and higher recovery cost (Zabed et al., 2014).
- Ethanol production is influenced by pH of the broth as it affects bacterial contamination, yeast growth, fermentation rate and by-product formation.
- The permeability of some essential nutrients into the cells is influenced by the concentration of H^+ in the fermentation broth (Zabed et al., 2014).
- Moreover, the survival and growth of yeasts is influenced by the pH in the range of 2.75–4.25 (Fleet and Heard, 1993).
- In fermentation for ethanol production, the optimum pH range of *S. cerevisiae* is 4.0–5.0 (Lin et al., 2012).
- When the pH was below than 4.0, a longer incubation period is required but the ethanol concentration was not reduced significantly.
- However, when then pH was above 5.0, the concentration of ethanol reduced substantially (Staniszewski et al., 2007).

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- Fermentation time affect the growth of microorganisms as shorter fermentation time causes inefficient fermentation due to inadequate growth of microorganisms.
- On the other hand, longer fermentation time gives toxic effect on microbial growth especially in batch mode due to the high concentration of ethanol in the fermented broth.
- Complete fermentation can be achieved at lower temperature by using longer fermentation time which results in lowest ethanol yield (Zabed et al., 2014).
- Agitation rate controls the permeability of nutrients from the fermentation broth to inside the cells and removal of ethanol from the cell to the fermentation broth.
- The greater the agitation rate, the higher the amount of ethanol produced.
- Besides, it increases the amount of sugar consumption and reduces the inhibition of ethanol on cells.
- The common agitation rate for fermentation by yeast cells is 150–200 rpm.
- Excess agitation rate is not suitable for smooth ethanol production as it causes limitation to the metabolic activities of the cells (Zabed et al., 2014).

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- Inoculum concentration does not give significant effects on the final ethanol concentration but it affects the consumption rate of sugar and ethanol productivity (Laopaiboon et al., 2007).
- The production of ethanol was seen to be increased with the increase in cell numbers from 1×10^4 to 1×10^7 cells per mL but there was no significant ethanol production found between 10^7 and 10^8 cells per mL.
- This is because the increase in cell concentration within certain range reduces fermentation time as the cells grow rapidly and directly consumes sugars into ethanol (Zabed et al., 2014).
- Overall, the ideal temperature for bioethanol production depends on the ideal temperature of the yeasts.
- Most of the fermenting medium used for bioethanol production has pH in the range of 4.5–5.5 with various sugar concentration.
- Fermentation process is commonly performed at 24 and 72 h with rotation at 120 and 150 rpm.
- The common inoculum size employed in bioethanol production are 5% and 10%.
- Zhang et al. (2011) reported the highest ethanol concentration (128.5g/L) and ethanol productivity (4.76g/L/h) probably due to favourable conditions for the yeast to produce bioethanol.

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Yeast strain	Feedstock	Pretreatment	Fermentation condition	Ethanol concentration (g/L)	Ethanol Productivity (g/L/h)
S. cerevisiae CHY1011	*Miscanthus sacchariflorus*	CHEMET with sodium hydroxide	Continuous SSF, 33 °C, 56 h, 25% WIS	69.2	1.24
S. cerevisiae TMB3400	Wood chips	Steam explosion	Batch SSCF, 3 °C, 96 h, 8% WIS	32.9	0.34
S. cerevisiae ATCC24858	Reed	Phosphoric acid-acetone	Batch SSF, 38 °C, 150 rpm, 96 h, 36.1% WIS	55.5	0.57
S. cerevisiae VIT C-10880	*Arundo donax*	Steam explosion	Enzymatic hydrolysis, 45 °C, 72 h; Batch SHF, 32 °C, 500 rpm, 96 h, 10% WIS	20.6	0.21
S. cerevisiae	Reed	Liquid hot water	Enzymatic hydrolysis, 50 °C, 18 h; Semi fed-batch SSF, 36 °C, 60 h, 10% WIS	39.4	0.66
S. cerevisiae TMB 3400	Wheat meal and wheat straw	Steam explosion	Enzymatic hydrolysis, 40 °C, 850 rpm, 120 h; Fed-batch SHCF, 32 °C, 300 rpm, 120 h, 7.5% WIS	53.3	0.44
S. cerevisiae	*Liriodendron tulipifera*	Acid-free organosolv	Batch SSF, 30 °C, 150 rpm, 96 h, 1% WIS	29.9	0.42
Baker's yeast	Corn stover	Steam explosion	Fed-batch SSF, 30 °C, 700 rpm, 72 h, 10% WIS	25.7	0.36
S. stipitis CBS 6054	*Miscanthus giganteus*	Dilute oxalic acid	SSF, 30 °C, 96 h, 10% WSF	12.1	0.13
S. cerevisiae	Industrial hemp	Steam explosion	SSF, 37 °C, 348 rpm, 72 h, 7.5% WIS	21.3	0.30

Processes involved in bioethanol production (Reproduced by Mohd Azhar et al., 2017)

Yeast strain	Feedstock	Temperature, (°C)	pH	Time (h)	Sugar concentration (g/L)	Agitation rate (rpm)	Inoculum size (%)	Ethanol concentration (g/L)	Ethanol Productivity (g/L/h)
Saccharomyces cerevisiae CHY1011	Cassava starch	32	4.5	66	585.0	120	5	89.1	2.10
Saccharomyces cerevisiae ZU-10	Corn stover	30	5.5	72	99.0	120	5	41.2	0.57
Saccharomyces cerevisiae K35	Instant noodle waste	30	–	24	84.0	250	5	41.3	1.72
Saccharomyces cerevisiae	Wood	30	5.5	16	37.47	150	10	18.52	1.16
Saccharomyces cerevisiae ATCC #24858	Reed	38	5.0	96	123.0	150	10	55.0	0.57
Saccharomyces cerevisiae	Sweet potato	30	5.3	24	240.0	150	7	128.5	4.76
Kluyveromyces marxianus K213	Water hyacinth	42	4.8	24	23.3	–	5	7.34	0.31
Kluyveromyces marxianus CECT 10875	Wheat straw	42	5.5	72	–	150	–	36.2	0.50
Saccharomyces cerevisiae GIM-2	Paper sludge	33	–	16	27.8	60	6	9.5	0.59
Saccharomyces cerevisiae CHFY0321	Cassava mash	33	–	42	183.5	100	5	86.1	2.41

Factors affecting bioethanol production (Reproduced by Mohd Azhar et al., 2017)

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5.0 BIOETHANOL SEPARATION AND PURIFICATION (DOWNSTREAM)

- Separation process is very crucial in bioethanol production as it consumes the highest energy in the process (Amornraksa et al., 2020).
- Several novel separation techniques have been developed to separate and purify ethanol more efficiently.
- In most cases, the distillation column is used as the critical method for separation due to its performance and reliability.
- However, the purification method used for ethanol dehydration may be various. The conventional method being used commercially is a molecular sieve by the adsorption process (Humbird et al., 2011).
- The principle of molecular sieve is based on the difference in molecular size between water and ethanol.
- Small molecules that can pass through the pores are adsorbed, while the larger molecules are not.
- Typically, the molecular sieve for ethanol dehydration has a pore diameter of 3A°, capable of adsorbing water that has a diameter of 2.5–2.8 A° but not ethanol that has a diameter of 4–4.4 A° (Kumar et al., 2010).

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- Besides the conventional molecular sieve, extractive distillation is another method that can be used to produce anhydrous ethanol.
- The extractive distillation involves two columns, which are extractive distillation column and recovery column (Bastidas et al., 2010).
- A relatively non-volatile liquid solvent such as ethylene glycol is used to change the relative volatilities of the components.
- In Meirelles et al. work (1992), extractive distillation using ethylene glycol as a solvent was used for ethanol dehydration.
- The experimental and simulation results showed that extractive distillation could be used to achieve high purity of ethanol with low energy consumption.
- A comparison of three ethanol dehydration techniques, including azeotropic distillation, extraction, and adsorption was studied Bastidas et al. (2010).

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- It was revealed that extractive distillation with ethylene glycol as a solvent is the best choice in terms of operation and economics.
- The successful use of extractive distillation for bioethanol production has also been demonstrated by Chuenbubpar et al. (2018).
- Pervaporation is another promising separation technique that can be used to produce anhydrous ethanol (Bermudez et al., 2014).
- Pervaporation is a kind of membrane separation processes where a liquid feed is separated into two streams, which are permeate and retentate (Kaminski et al., 2008).
- The water passes through the membrane as vapor permeate, while the ethanol remains in the liquid phase as retentate.
- The driving force of pervaporation is a pressure difference created over the membrane.
- The vacuum is located on the permeate side while atmospheric pressure is operated on the feed side, causing the pressure difference.

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- Typical steps in the bioethanol production from lignocellulose-containing raw materials are as follow (Arijana et al., 2018) :
  - i) pretreatment of cellulose and hemicellulose to become more accessible in the subsequent steps;
  - ii) acid or enzymatic hydrolysis of polysaccharides into simple sugars;
  - iii) microbial fermentation of the simple sugars (hexoses and pentoses) to ethanol; and
  - iv) separation and concentration of bioethanol
- Two energy-demanding separation steps are necessary to obtain purified ethanol (95.63 % by mass) from binary azeotrope ethanol-water (Huang et al., 2008).
- The first step is a standard distillation that concentrates ethanol up to the level of 92.4–94 % by mass.
- The cyclic distillation for ethanol purification is an energy-efficient alternative that is characterised by relatively low investments.
- The second step involves ethanol dehydration to obtain an anhydrous ethanol (ethanol concentrations above the azeotropic composition).

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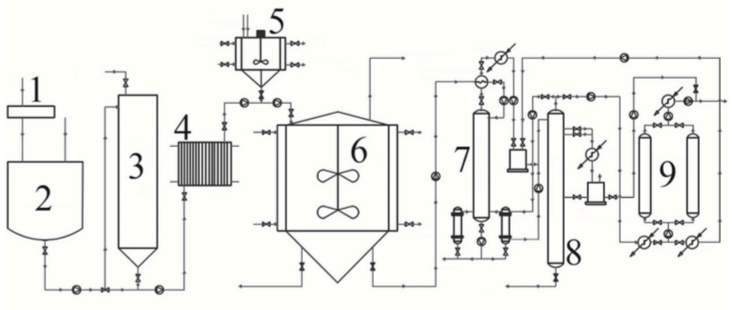

The scheme of bioethanol production from lignocellulose containing raw materials. 1=milling, 2=pretreatment, 3=saccharification, 4=heat exchanger, 5=propagator (inoculum seeding tank), 6=bioreactor, 7=stripping column, 8=rectifying column, 9=molecular sieves (Reproduce from Arijana et al., 2018)

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- In order to reduce energy consumption of conventional distillation, membrane techniques have gained attention as an alternative because of a number of advantages that make them attractive for the separation of liquid mixtures.
- They have high separation efficiency, energy and operating costs are relatively low, they produce no waste streams, and they can be used in the separation of temperature-sensitive materials (Hwang et al., 2014).
- Among the available membrane techniques, pervaporation is quite attractive due to its simplicity, low energy-demands and the absence of extra chemicals; besides, the vacuum part of the process consumes the majority of energy (Huang et al., 2008).
- It uses a non-porous membrane which separates the mixture as a result of molecular interactions between the feed components and the membrane.
- The transport of molecules through the membrane generally involves three steps: (i) molecules from the feed are selectively adsorbed into the membrane, (ii) diffusion of the adsorbed molecules across the membrane, and (iii) desorption of the molecules into the gas phase on the permeate side.

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- Pervaporation can be carried out in parallel to the fermentation.
- This is promising system for in situ extraction of ethanol, which is harmless to the working microorganism (Kaewkannetra et al., 2011).
- Therefore, low ethanol medium concentrations can prevent ethanol inhibition, and consequently the bioprocess can run continuously.
- Before the pervaporation unit, a microfiltration/ultrafiltration module has to be installed for biomass removal to prevent deterioration of the pervaporation membrane.
- This integrated system was used in the study of ethanol separation from aqueous solution and fermented sorghum juice (Kaewkannetra et al., 2011).
- Amornraksa et al. (2020) systematically designed and simulated 3 types of separation process for bioethanol production from corn stover by using Aspen Plus software guided by thermodynamic insight.
- The conventional molecular sieve (Case 1) was compared with the extractive distillation (Case 2) and pervaporation membrane (Case 3).

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- It was revealed by Amornraksa et al. (2020) that pervaporation membrane (Case 3) is the best process in all aspects.
- Its energy consumption and carbon footprint by Case 3 are 60.8% and 68.3%, respectively which is 6.6% and 24.2% lower than Case 1.
- The carbon footprint in Case 3 was found to be the lowest, at 0.575 CO_2 eq. that is mostly generated from the distillation column that separates wastewater.
- The design of the pervaporation process is less complicated than the other cases, resulting in the lowest total capital cost and total production cost at 37.0 and 9.88%, respectively.
- Even though pervaporation was found to be the best separation process, more works can be done to improve the process performance.
- One potential improvement is to recover the ethanol loss to wastewater at the pervaporation.
- It may be done by installing a distillation column to separate water from the wastewater and send back the concentrated ethanol to the pervaporator to recover more ethanol.

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- Overall, from the standpoint of industrial application, bioethanol removal from fermentation broths via pervaporation membrane is still rather limited (Ping et al., 2021).
- The current research status is that most of the pervaporation membrane experiments reported have been conducted at laboratory scales and performed with simple and ideal binary aqueous solutions.
- Thus resulting in a lack of understanding on pervaporation membrane performance in real fermentation condition using broths.
- Long-term trial evaluation of pervaporation membrane is preferable to be implemented at actual industrial conditions where pervaporation membrane integrates with fermentor.
- In addition, more efforts should be focused on enhancing the long-term operation stability of membranes including selectivity, chemical and temperature resistance, and robustness, in particular polymeric and mixed matrix membranes.
- Finally, it is expected that more and more successful research could be realized into commercial products and this separation process will be deployed in industrial practices in the near future (Ping et al., 2021).

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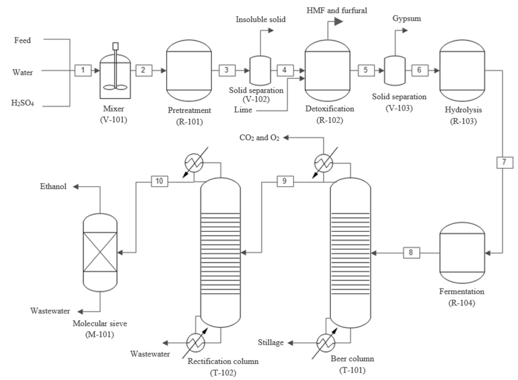

The production of ethanol production process from corn stover – Case 1 (Conventional molecular sieve) (Amornraksa et al., 2020)

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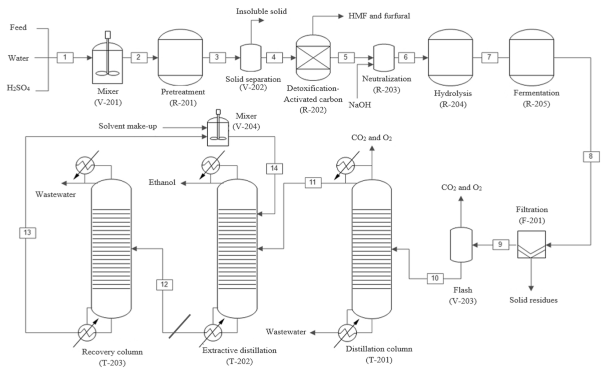

The production of ethanol production process from corn stover – Case 2 (Extractive distillation) (Amornraksa et al., 2020)

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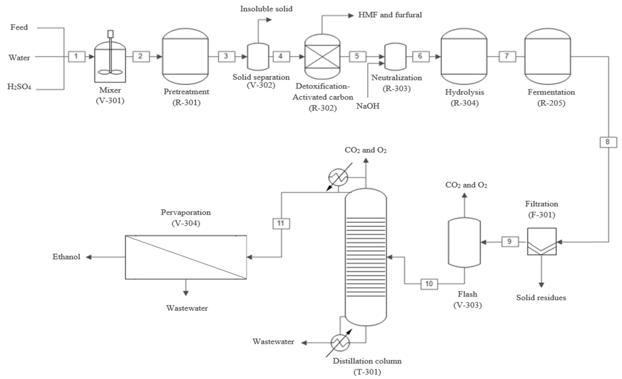

The production of ethanol production process from corn stover – Case 3 (Pervaporation Membrane) (Amornraksa et al., 2020)

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6.0 BIOETHANOL QUALITY CONTROL

- Although the bioethanol industry is not regulated to the same extent as the food or pharmaceutical sectors, Ebert (2009) has discussed voluntary quality assurance models for fuel ethanol production plants, based on:
  - ISO (International Organisation for Standardisation)
  - HACCP (Hazard Analysis and Critical Control Points)
  - USDA PVP (United States Department of Agriculture Process Verification Program)
- Quality control monitoring for individual bioethanol plants, based on teamwork and accurate statistical analyses of process data, is essential to boost profitability and maintain competitiveness (Walker, 2010).
- In addition to ensuring quality of bioethanol processes, quality parameters of the end product are also important.
- In the US, The American Society for Testing and Materials International (ASTM) approves analytical specifications for bioethanol transportation fuel performance quality (Davis, 2009).
- This includes the key parameters to be measured, their units of measurement and their influence on quality.

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- For example, pH and water elimination are important parameters for internal combustion engines.
- The Renewable Fuels Association (RFA) recommend minimum testing frequencies and methods for bioethanol to ensure product quality and consistency and to meet ASTM standards.
- Table below provides an example of ASTM specification for denatured fuel ethanol and E85 (Walker, 2010).

Quality parameter	Limits for denatured fuel ethanol	Limits for E85
Ethanol, %v/v min	92.1	74*
Methanol, %v/v max	0.5	0.5
Water, %v/v max	1.0	1.0
Acidity (as acetic acid), mass% (mg/L) max	0.007 (56)	0.005 (40)
pHe	6.5-9.0	6.5-9.0
Copper, mg/kg max	0.1	0.07
Inorganic chloride, mass ppm (mg/ l) max	40 (32)	1 (mg/kg)
Solvent-washed gum, mg/L max	5.0	5.0
Sulphur, mass ppm max	30	
Sulphate, mass ppm max	4	
Denaturant, %v/v	1.96 (min); 5.0 (max)	
Hydrocarbon/aliphatic ether, %v/v		17-26
Appearance	Clear and bright, visibly free of suspended or precipitated matter	Clear and bright, visibly free of suspended or precipitated matter

*plus higher alcohols

ASTM specification for denatured fuel ethanol and E85

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- ASTM publish standards with specifications for the following bioethanol products:
  - –ASTM D 4806-07 (Standard specification for denatured fuel ethanol for blending with gasolines for use as automotive spark-ignition engine fuel).
  - –ASTM D 5798-07 (Standard specification for fuel ethanol (Ed75-Ed85) for automotive spark- ignition engines)
- These specifications are updated regularly (www.astm.org).
- The specification for denatured ethanol defines the acceptable and unacceptable hydrocarbon denaturants and these are also regulated by the US Alcohol and Tobacco Tax and Trade Bureau (TTB) to ensure bioethanol is unfit for human consumption.
- In most countries, bioethanol is blended with petrol at proportions of 2-10%, the current exception being Brazil where all gasoline used contains 20-25% ethanol (E20, E25).
- For blending with petrol, bioethanol requires to be anhydrous.
- Flexible fuel vehicles (FFVs) have an internal combustion engine and are capable of operating on petrol and any blend of petrol and ethanol up to 83%.
- E85 (or flex fuel) is a petrol-ethanol blend containing 51% to 83% ethanol, depending on geography and season.

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7.0 BIOETHANOL FUTURE PROSPECT

- We have discussed the first-generation and second-generation of bioethanol production.
- The third-generation of bioethanol is using biomass from micro/macroalgea biomass while the fourth-generation is from genetically modified cynobacteria through 'photofermentation' (direct conversion of light and CO_2 into ethanol).
- Bioethanol generation exploiting the lignocellulosic biomass can reduce its production cost as it is an inexpensive biomass waste available abundantly throughout the year.
- In order to develop the commercial production of bioethanol, the process economics of cellulosic ethanol production must be improved.
- In addition, pervaporation membrane looks promising when integrates with fermentor to intensify the separation and purification processes.

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7.0 BIOETHANOL FUTURE PROSPECT

- It is expected that the production of bioethanol from lignocellulosic biomass with improvement on the separation process using pervaporation membrane might be the important solution in finding a suitable substitution for fossil fuels as energy resources.
- Thus, bioethanol can be easily commercialized as the product cost only depends on the technology involved in the production which promotes a sustainable economic development.
- Economic growth, crisis, and security in the country, therefore, can be strengthened as economically sustainable fuels produced can lower the dependency on imported fuels in the country.

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