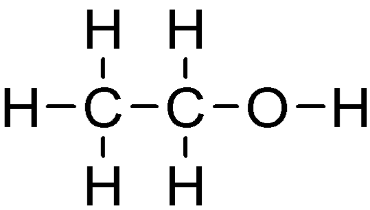
Contents

Ethanol

Main used Microorganisms for ethanol production:

- Yeast: *Saccharomyces cerevisiae; Pichia stipidis*
- *Bacteria: Zytomonas mobilis*; *Escherichia coli*

Currently global ethanol production primarily from sugar and s

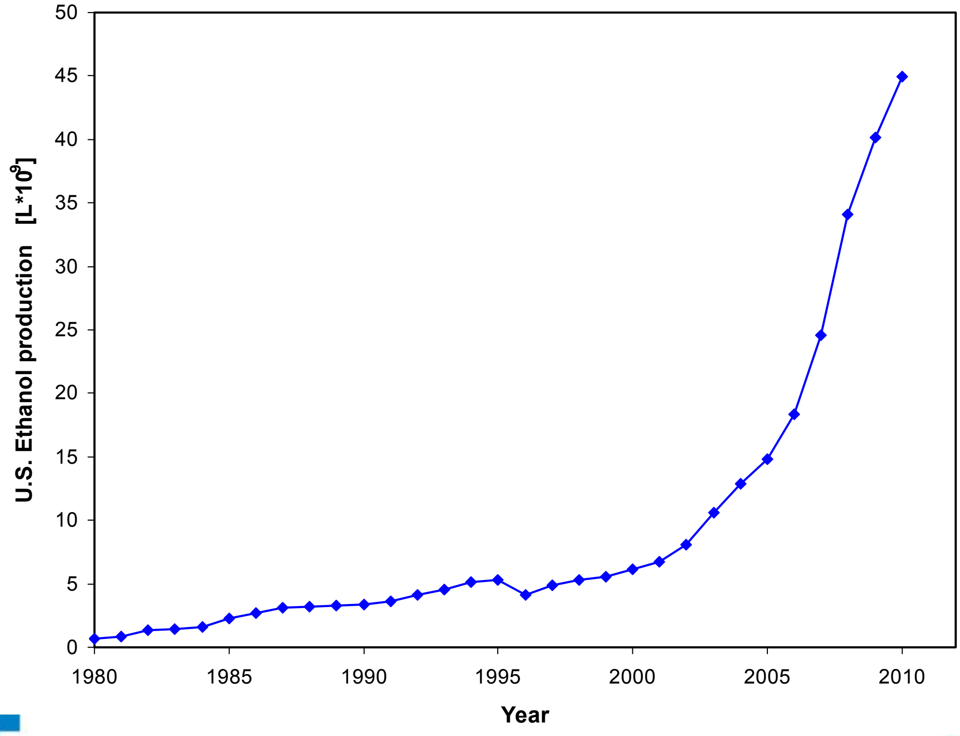


Bioethanol plant in Wanze from BioWanze, Belgium

Properties

Molecular formula	C_2H_6O
Molar mass	46.07 g mol^{-1}
Density	0.789 g cm^{-3}
Melting point	−114 °C, 159 K
Boiling point	78 °C, 351 K

Ethanol production in the U.S.



from RFA – Renewable Fuels Association; http://www.ethanolrfa.org/

History of fermentative produced beverages

Rice wine in China

Wine and beer in the Sumerian city-states[3]

Kombucha

Herb wine in Egypt [2]

Kombucha fermented tea from China

Traditionell drinking of wine in the chinese province Yunnan[1]

Early illustration of Sumerian beer drinkers [2]

ca. 5000 B.C. ca. 3500 B.C. ca. 3150 B.C. ca. 220 v. Chr.

from [1] Thadeusz, 2009, Am Anfang war der Sud; *Der Spiegel*, 52; [2] McGovern et al.,2009, Ancient Egyptian herbal wines; *PNAS,* 106 (18):7361–7366; [3] Michel et al., 1993, The first beer and wine; *Aanalytical Chemistry, 65 (8):408-413;*

History of fermentative produced beverages

Ayran invented in Turkey

Oldest brewery in the world: Bayerische Staatsbrauerei Weihenstephan

Monastary Weihenstephan

German (Bavarian) purity law

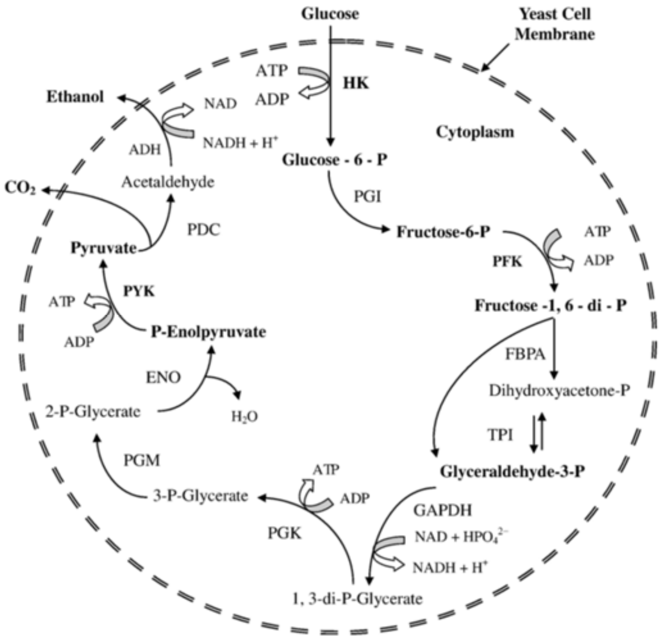
ca. 1000 A.D. | 1040 A.D. | 1516 A.D.

Bioethanol fuel blends

Introduction of bioethanol fuel blends:

- All European states had to increase the allowed max. ethanol contend in fuels from 5 % (E5) to 10 % (E10) since end of 2010 (usage not mandatory)
- USA introduction of E10 in 2007 (mandatory in 10 states, e.g. Florida, Hawaii etc.)
- USA are discussing and planning to increase to E15 since 2007
- Brasilia uses E20-E25
- Sweden, Thailand and Brasilia allow usage of E85

Metabolic pathway of ethanol fermentation in *S. cerevisiae*



Abbreviations:

HK: hexokinase,

PGI: phosphoglucoisomerase,

PFK: phosphofructokinase,

FBPA: fructose bisphosphate aldolase,

TPI: triose phosphate isomerase,

GAPDH: glyceraldehydes-3-phosphate dehydrogenase,

PGK: phosphoglycerate kinase,

PGM: phosphoglyceromutase,

ENO: enolase,

PYK: pyruvate kinase,

PDC: pyruvate decarboxylase,

ADH: alcohol dehydrogenase.

from Bai 2008; Biotechnology Advances 26, p. 89–105

Ethanol fermentation with *S. cerevisiae*

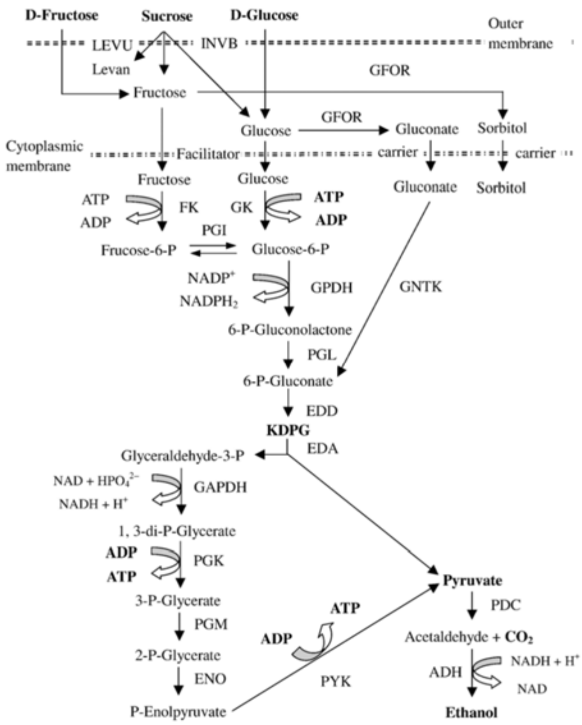
Anaerobic fermentation

- Glycolysis (Embden-Meyerhof-Parnas (EMP) pathway) is main metabolic pathway for ethanol production in *S. cerevisiae*
- ATPs produced in glycolysis are used for cell growth (yeast cells as co-product of ethanol fermentation)
- Ethanol production linked to growth. In yeast immobilization (gel entrapment) the cells do not grow ➡ Immobilization not feasible

By-products:

- Glycerol up to 1.0% (w/v)
- Organic acids
- Higher alcohols

Carbohydrate metabolic pathways in *Zymomonas mobilis*



Abbreviations:

LEVU: levansucrase

INVB: invertase

GFOR: glucose-fructose oxidoreductase

FK: fructokinase

GK: glucokinase

GPDH: glucose-6-phosphate dehydrogenase

PGL: phosphogluconolactonase

EDD: 6-phosphogluconate dehydratase

KDPG: 2-keto-3-deoxy-6-phosphogluconate

EDA: 2-keto-3-deoxy-gluconate aldolase

GNTK: gluconate kinase.

from Bai 2008; Biotechnology Advances 26, p. 89–105

Ethanol fermentation with *Zymomonas mobilis*

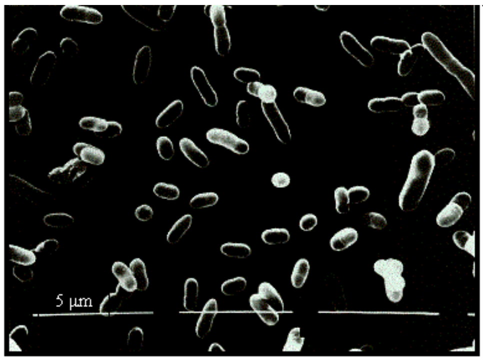
Zymomonas mobilis an effective ethanol producer

Anaerobic, gram-negative bacterium

Originally discovered in fermenting sugar-rich plant saps e.g. the traditional drink of Mexico pulque (alcoholic brewarage from maguey)

Entner-Doudoroff (ED) pathway for production of ethanol from glucose

- Produces only one ATP for growth (compared to two in glycolysis) and produces less biomass



from Bai 2008; Biotechnology Advances 26, p. 89–105

Drawbacks of ethanol production with *Z. mobilis*

Specific substrate spectrum of *Z. mobilis*

- Uses only D-glucose, D-fructose and sucrose as C-source
- Growth on sucrose produces fructose oligomeres and reduces ethanol yield
- ➢ Unsuitable for ethanol production from molasses due to narrow substrate range
- ➢ Unsuitable for ethanol production from starch as only glucose is effectively used from the products from hydrolysis (maltose, sucrose, fructose)

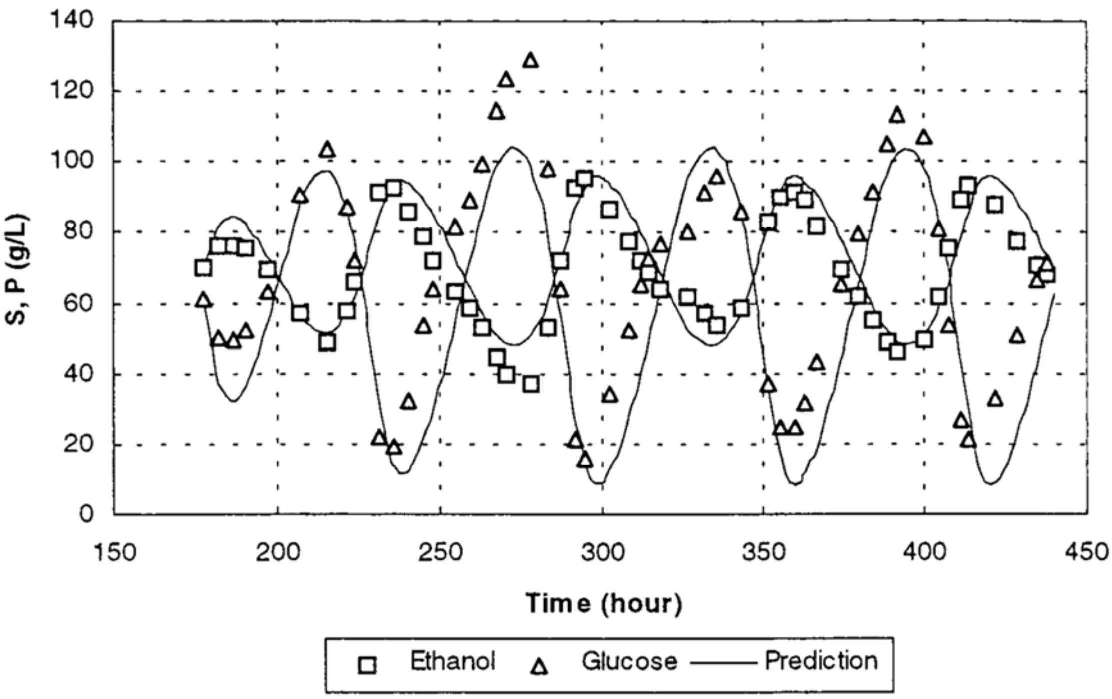
Can not be used as animal feed like *S. cerevisiae* (biomass disposal)

Continuous fermentation tends to be oscillatory under certain conditions

- ➢ reducing the ethanol yield

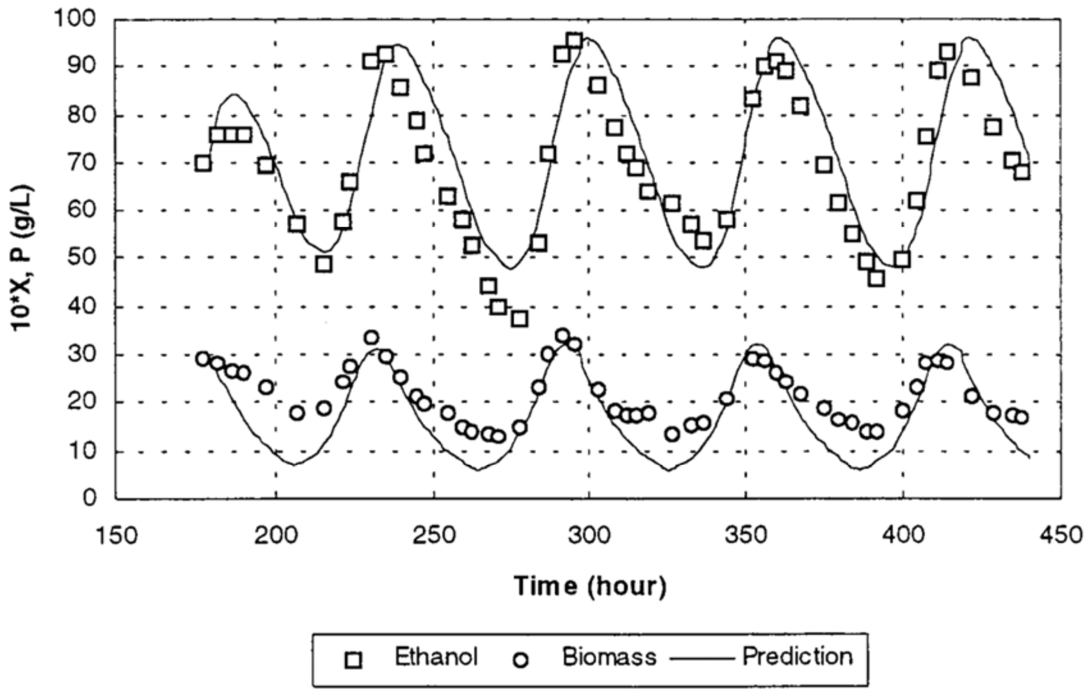
from Bai 2008; Biotechnology Advances 26, p. 89–105

Sustained oscillations in fermentation of *Z. mobilis*



from McLellan 1999; *Biotechnol. Prog.* 1999, *15*, 667-680

Sustained oscillations in fermentation of *Z. mobilis*



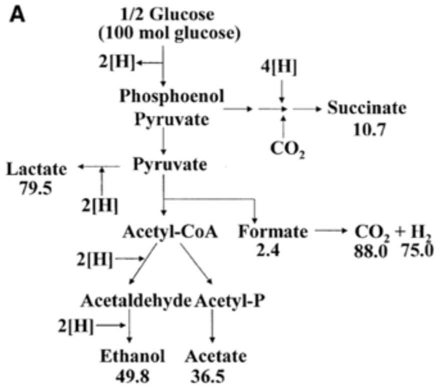
Sustained oscillations in fermentation of *Z. mobilis*

Reason of oscillatory behavior of *Z. mobilis* in continuous cultures

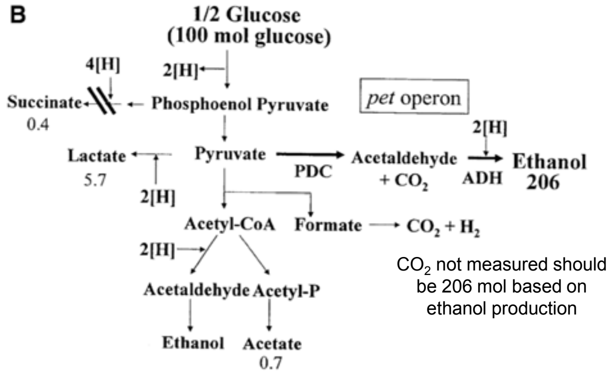
- Cultures with high glucose feed concentrations and low dilution rates
- Uncoupled growth and ethanol product formation
- Growth rates are highly ethanol inhibited and demonstrate dynamic and instantaneous behavior
- Periodic swings between filamentous and unicellular morphologies during oscillation caused be changing stress levels (ethanol concentration change rates)

Carbohydrate metabolic pathways in *E. coli* for ethanol production

Metabolic pathway in *E. coli* K12 (wild type)



Metabolic pathway in *E. coli* KO11 (genatically modified)

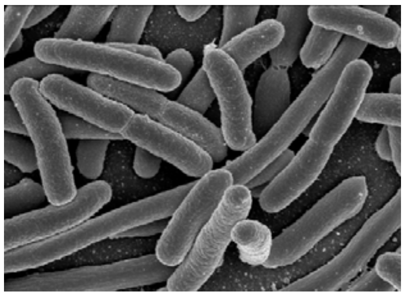


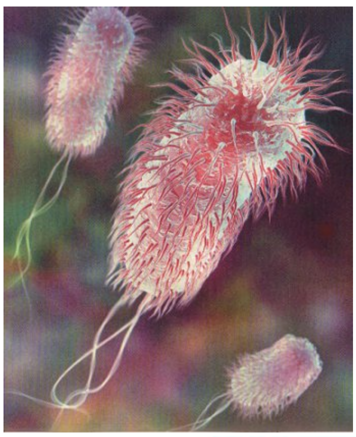
Gentetic engineering of *E. coli* for ethanol production:

- Introduction of PDC (pyruvate decarboxylase) and ADH (alcohol dehydrogenase) from *Z. mobilis* inside the artificial operon *pet*
- Interruption of gene *frd* (fumarate reductase) to reduce succinate production

from Dien 2003; Appl. Microbiol. Biotechnol. 63: 258–266; Ohta 1991, Applied and environmental Microbiology, Apr. 1991, p. 893-900

Ethanol fermentation with *E. coli*





Escherichia coli, a facultative anaerobic, gram-negative bacterium

Genetically modified to produce high amounts of ethanol

Wide substrate sugars spectrum (e.g. xylose, arabinose etc.)

Needs no complex growth factors

Major disadvantages of *E. coli*:

- Narrow and neutral pH growth range (ca. pH 5.8–8.0)
- Less robust cultures compared to yeast
- Negative public perceptions of *E. coli* strains

Comparison *S. cerevisiae, Z. mobilis and E. coli*

S. cerevisiae

Pros

- Generally Regarded As Save (GRAS)
- Usage as animal feed
- Long cultivation tradition

Cons

- Lower ethanol yield
- Can not use xylose as substrate (without genetic modification)
- Temp. max. 35°C

Z. mobilis

Pros

- GRAS
- High ethanol yield and productivity

Cons

- Limited substrate spectrum
- Can not be used as animal feed
- Continuous cultures tent to be oscillatory

E. coli

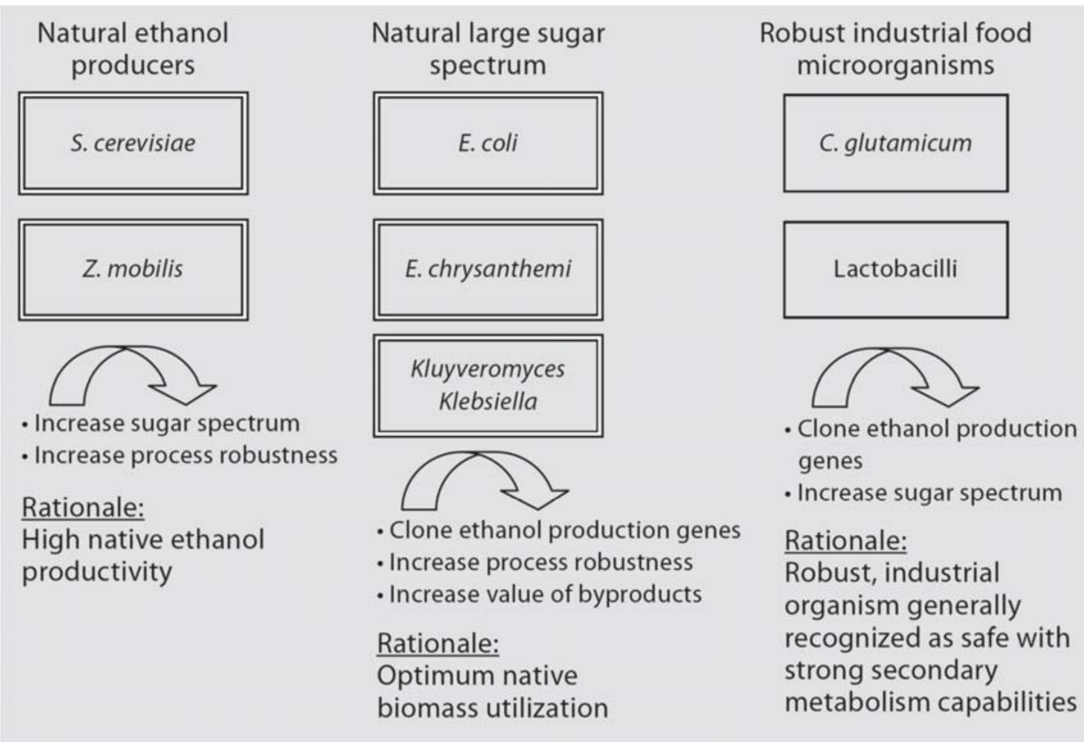
Pros

- Broad substrate spectrum
- High ethanol yields

Cons

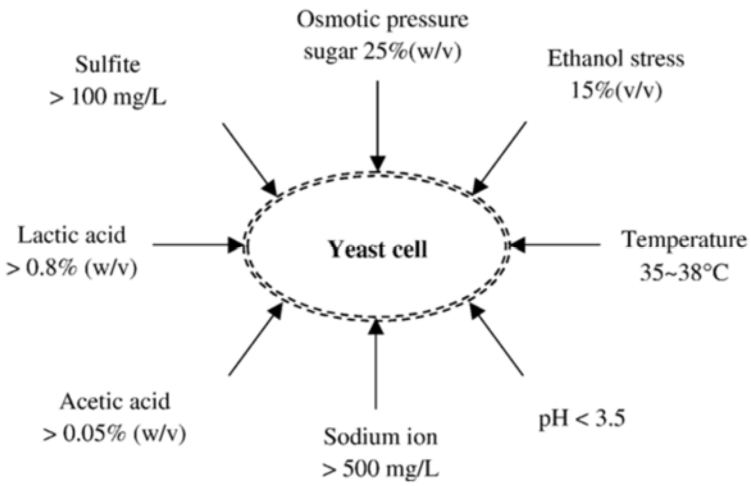
- Neutral pH range
- Genetically modified
- Not GRAS
- Can not be used as animal feed

Ethanol fermentation with different microorganisms



from Vertès 1999; J Mol Microbiol Biotechnol 15:16–30

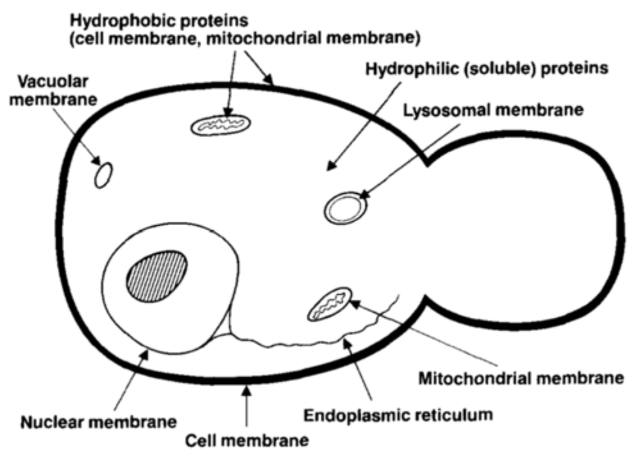
Potential environmental stresses on *S. cerevisiae* ethanol fermentation



Additional synergistic effects increase inhibition of ethanol production

Especially challenging in continuous processes (e.g. contamination produces acetate or lactate)

Possible target sites for ethanol inhibition in yeast cells



Glycolytic pathway

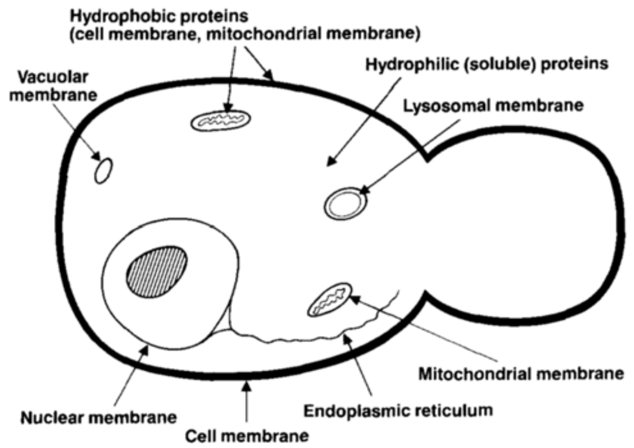
• inhibition of different enzymes (e.g. hexokinase and alcohol dehydrogenase)

Cell and organelles membranes

• main targets of ethanol inhibition

• fermentation by-products (e.g. acetaldehyde and acetate) exacerbate ethanol inhibition

Possible target sites for ethanol inhibition in yeast cells



Cell and organelles membranes

- decrease of fluidity of the membranes
  - unsaturated fatty acids (e.g. oleic acid (C18:1)) counter act this effect
  - small amount of O_2 necessary for production of unsaturated fatty acids
- Nutrient uptake affected by decreased activity of plasma membrane ATPase
  - trans membrane proton reduced
  - H^+ concentration in cells increase to unfavorable conditions

from Bai 2008; Biotechnology Advances 26, p. 89–105; D'Amore 1987; Enzyme Microb. Technol., 1987, vol. 9, June

Enzymatic hydrolysis of starch for ethanol production

Starch is one of the main substrates for ethanol production

Starch is entirely composed of α-D-glucose (glucopyranose) in two structures:

- Amylose a linear polymer of glucopyranose units linked through α-D-1,4 linkages
- Amylopectin a branched polymer containing chains with short degree of polymerization (DP = 20-25 glucopyranose residues) linked to C-6 of certain glucose moieties via α-D-1.6 linkages

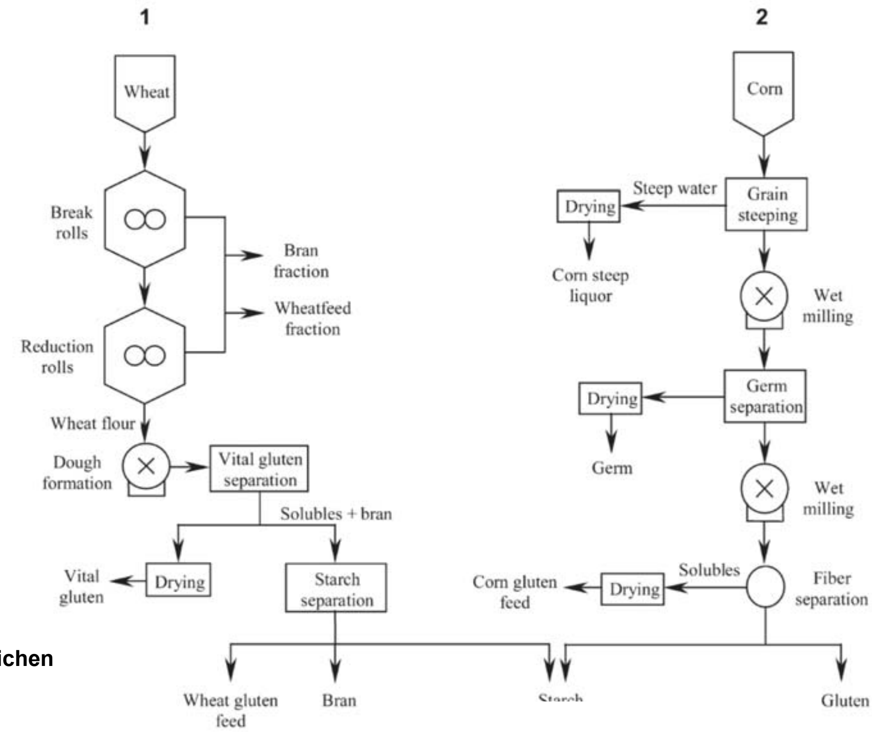
For ethanol production starch is hydrolyzed to glucose by amylolytic enzymes (α-amylase and glucoamylase)

$$\underset{\text{Starch}}{H(C_6H_{10}O_5)_nOH} \xrightarrow[\text{water}]{\text{amylolytic enzymes}} \underset{\text{Glucose}}{n\,C_6H_{12}O_6} \xrightarrow{\text{yeast}} \underset{\text{Ethanol}}{2n\,CH_3CH_2OH} + \underset{\text{Carbon dioxide}}{2n\,CO_2}$$

from Ingledew 1999; The alcohol textbook. 3rd ed. UK: Nottingham University Press

An ethanol plant in Colorado, surrounded by fields of corn

Schematic diagram of current processing of wheat and corn for producing fermentation media (1)



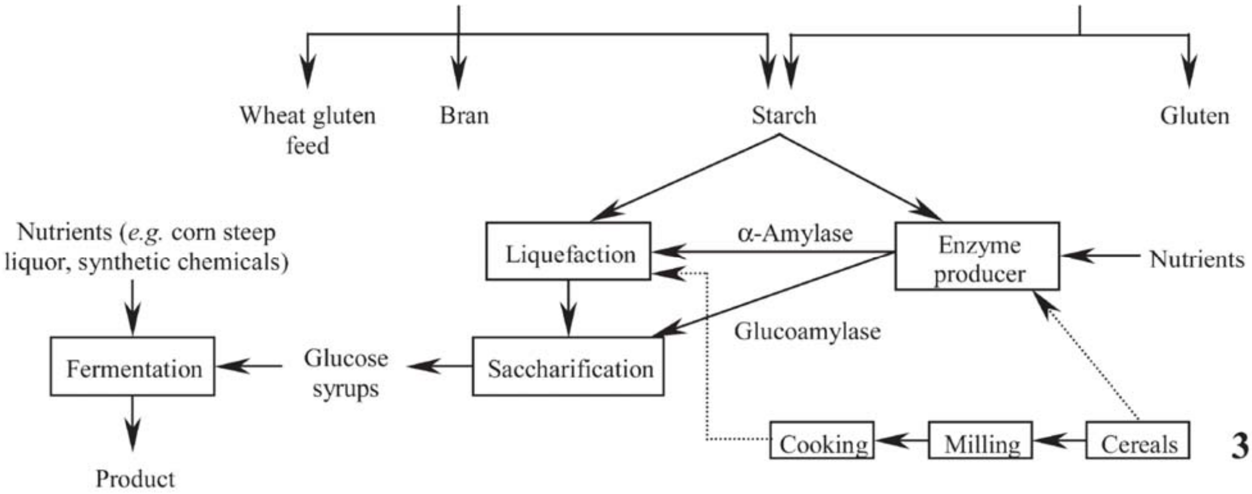
Bran = Kleie

Steeping = Einweichen

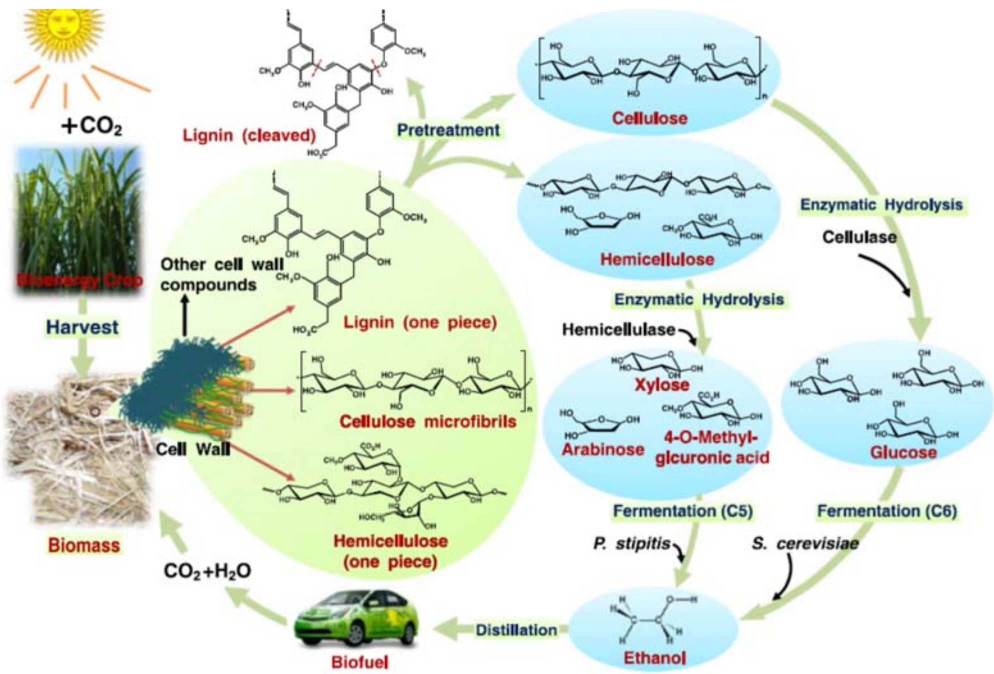
Germ = Keim

from Webb 2004; Adv Biochem Engin/Biotechnol 86: 195–268

Schematic diagram of current processing of wheat and corn for producing fermentation media (2)



Possible pathways for bioethanol fermentation from cellulosic feedstock



Composition of some lignocellulosic raw materials

(% of dry matter)

Raw material	Glucan	Mannan	Galactan	Xylan	Arabinan	Lignin	Ref
Agricultural residues							
Corn stover	36.4	0.6	1.0	18.0	3.0	16.6	[151]
Rice straw	34.2	-	-	24.5	-	11.9	[151]
Sugar cane bagasse	40.2	0.5	1.4	22.5	2.0	25.2	[152]
Wheat straw	38.2	0.3	0.7	21.2	2.5	23.4	[151]
Switch grass	31.0	0.3	0.9	20.4	2.8	17.6	[151]
Hardwood							
Salix	41.5	3.0	2.1	15.0	1.8	25.2	[153]
Softwood							
Pine	46.4	11.7	-	8.8	2.4	29.4	[151]
Spruce	49.9	12.3	2.3	5.3	1.7	28.7	[35]

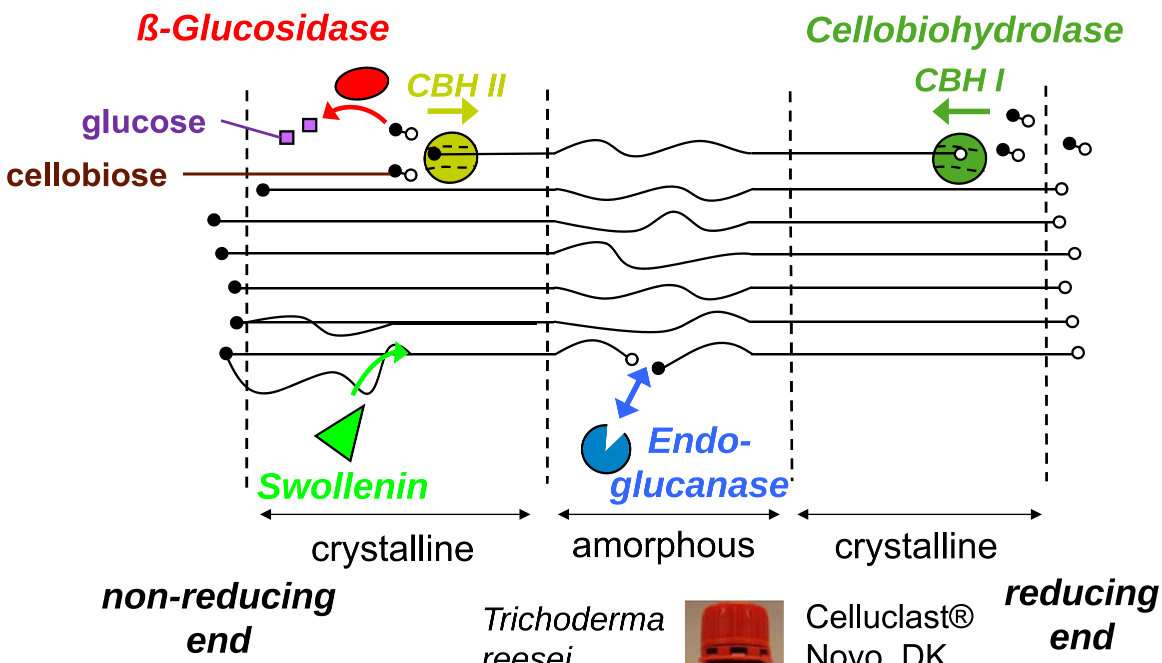
Salix = Grauweide

Pine = Kiefer

Spruce = Fichte

from Olofsson 2008; *Biotechnology for Biofuels* 2008, 1:7

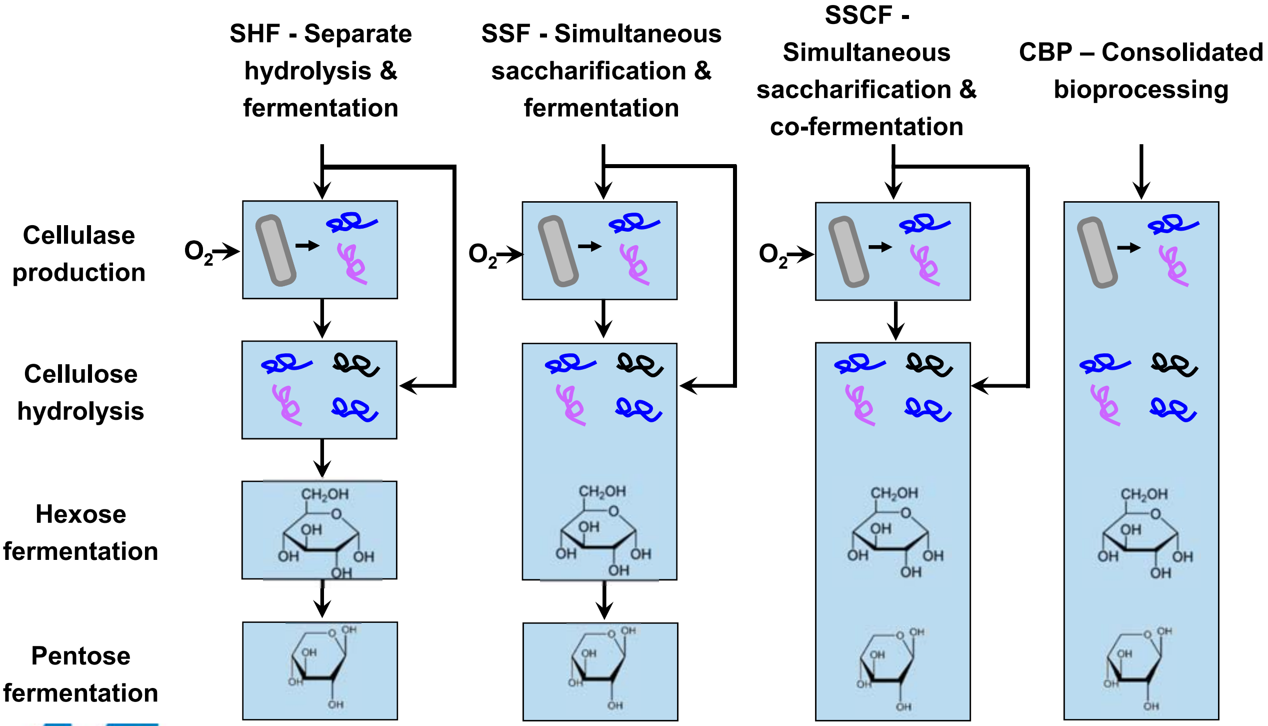
Cellulose degradation by *Trichoderma reesei* cellulases



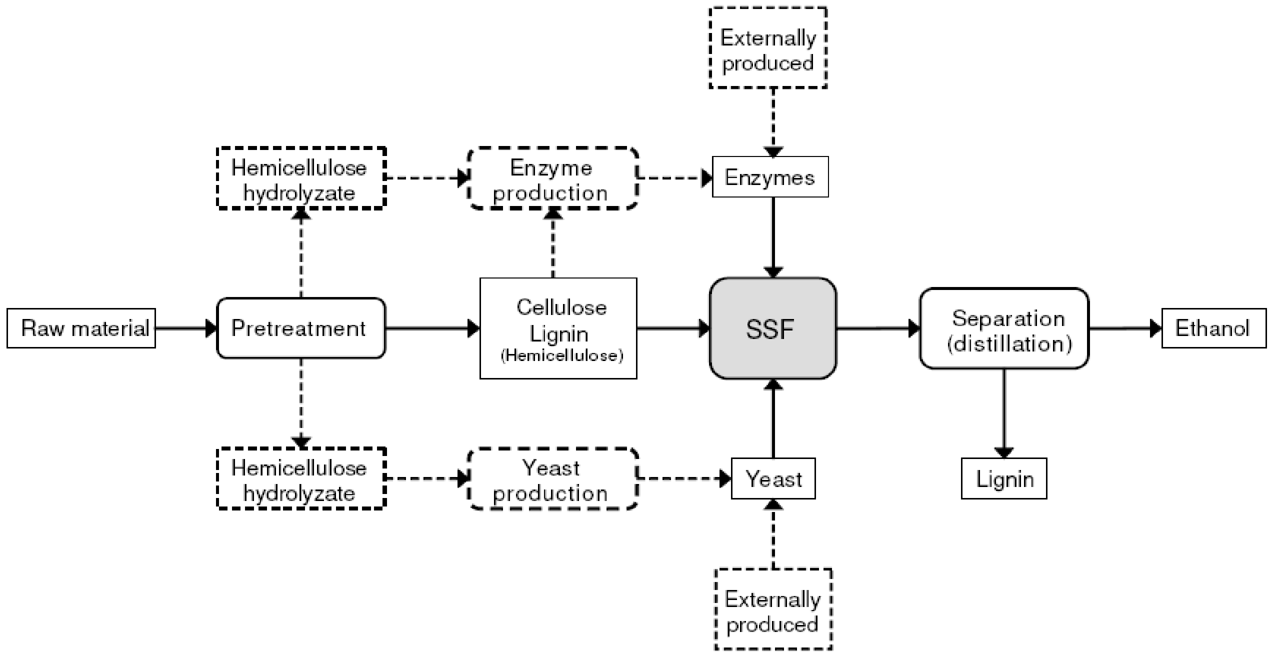
Trichoderma reesei

Celluclast® Novo, DK

Biomass processing using enzymatic hydrolysis and fermentation

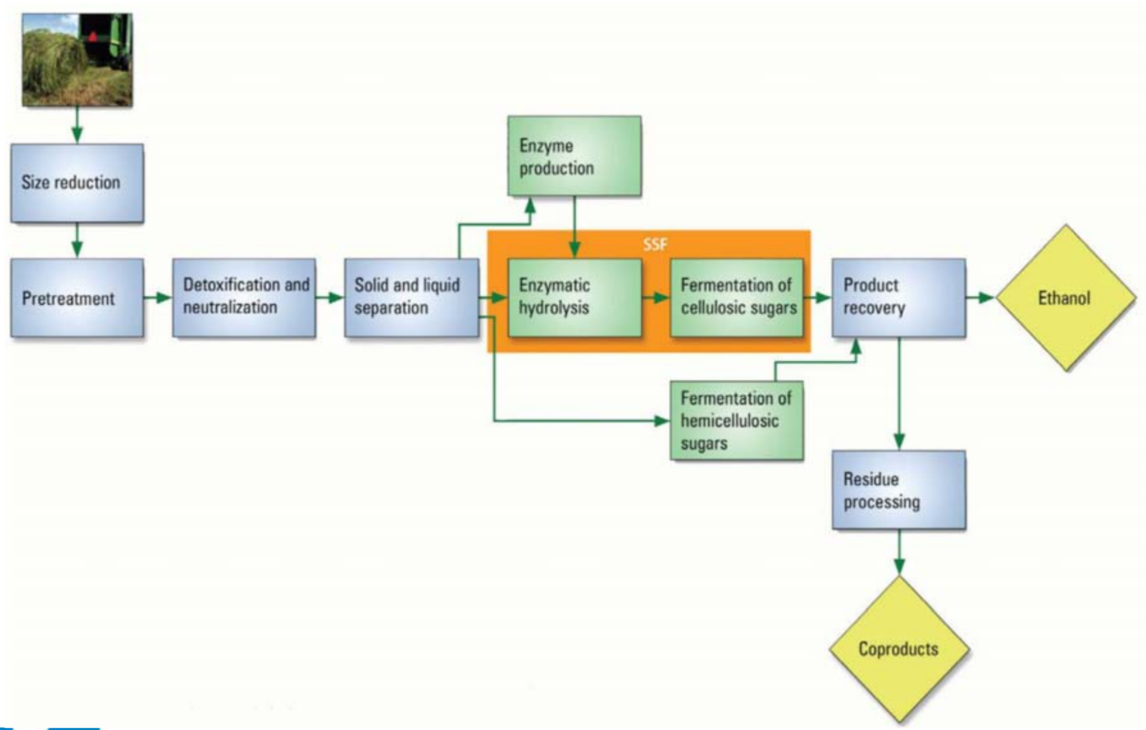


Schematic representation of an SSF process



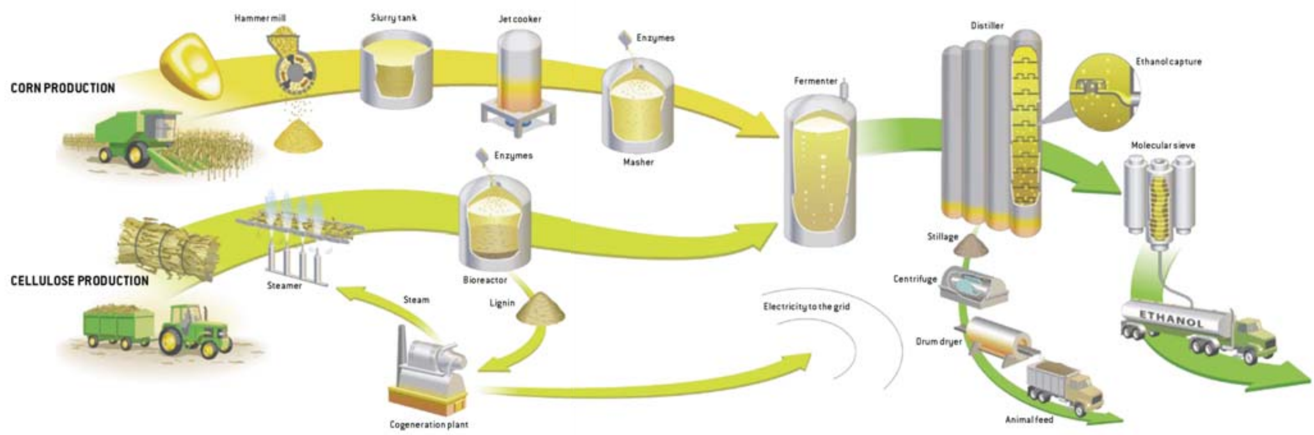
Steps in cellulosic ethanol production

From: Breaking the Biological Barriers to Cellulosic Ethanol



Difference of starch and lignocellulose biorefinery

The initial steps in converting corn or cellulose into ethanol differ significantly. Corn is ground, cooked and mashed before entering a fermenter. Cellulose is steamed to expose fibers that enzymes then convert into sugars in a bioreactor. Companies are still looking for bioreactions that are efficient on a large scale, but one payoff is the lignin that remains behind, which can be burned to cogenerate steam and electricity. The distillation of either raw material creates stillage, a valuable by-product that can be processed into animal feed.



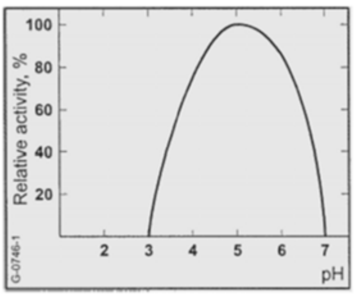
Ethanol production from D-xylose by various yeasts

Microorganisms	Temp. (°C)	Xylose (g l^{-1})	EOH (g l^{-1})	Ethanol Yield (g g^{-1})	Reference
Brettanomyces					
B. clausenii	MR	20.0*	21.4	0.43**	Parekh *et al.* (1988)
Candida					
C. tropicalis ATCC 1369	MR	100.0	5.4	0.11	Jeffries (1981)
C. shehatae NCL-3501	MR	10–80	NA	0.4–0.43	Abbi *et al.* (1996)
C. shehatae FPL-702	MR	NA	35.0	NA	Sreenath & Jefferies (1996)
Candida sp. XF-217	MR	100.0	30.0	0.42	Gong *et al.* (1981)
Clavispora					
Clavispora sp. 83-877-1	25.0	60.0	10.9	0.29	Nigam *et al.* (1985)
Kluyveromyces					
K. cellobiovorus KY 5199	28.0	100.0	30.0	0.31	Morikawa *et al.* (1985)
K. marxianus SUB-80-S	30.0	20.0	5.6	0.28	Margaritis & Bajpai (1982)
K. marxianus 83-SM16-10	25.0	20.0	5.2	0.26	Margaritis & Bajpai (1982)
K. marxianus IMB1,2,3,4&5	45.0	10.0	0.8–1.2	0.08–0.12	Banat & Marchant (1995)
Pachysolen					
P. tannophilus NRRL Y-2460	MR	20.0	5.3	0.27	Schneider *et al.* (1981)
P. tannophilus NRRL Y-2460	MR	115.0	23.0	0.30	Slininger *et al.* (1982)
P. tannophilus IFGB 0101	MR	30.0	3.8	0.13	Debus *et al.* (1983)
Pichia					
P. stipitis NRRL Y-5773	MR	30.0	12.0	0.36	Dellweg *et al.* (1984)
Schizosaccharomyces					
S. pombe	30.0	100.0	37.0	NA	Err-Cheng *et al.* (1986)

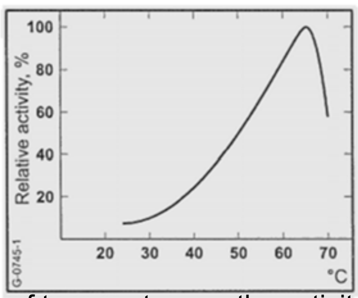
MR = mesophilic range; Ethanol yields = g ethanol produced/g substrate consumed; NA = none available.
*20 g xylose in 70 g total wood sugar; ** ethanol yield is calculated in reference to total sugar.

from Banat et al. 1998; World Journal of Microbiology & Biotechnology 14, 809±821

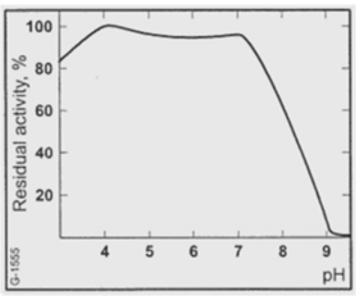
Different factors influencing SSF performance



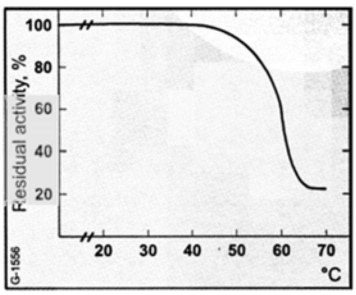
Influence of pH on the activity of celluclast
Enzyme conc.: 0.009 EGU/mL; Temp.: 50°C; Reaction time: 20 min



Influence of temperature on the activity of celluclast
Enzyme conc.: 0.009 EGU/mL; pH: 4.8; Reaction time: 20 min



Influence of pH on the stability of celluclast
Enzyme conc.: 0.9 EGU/mL; Temp.: 25°C; Reaction time: 16 h

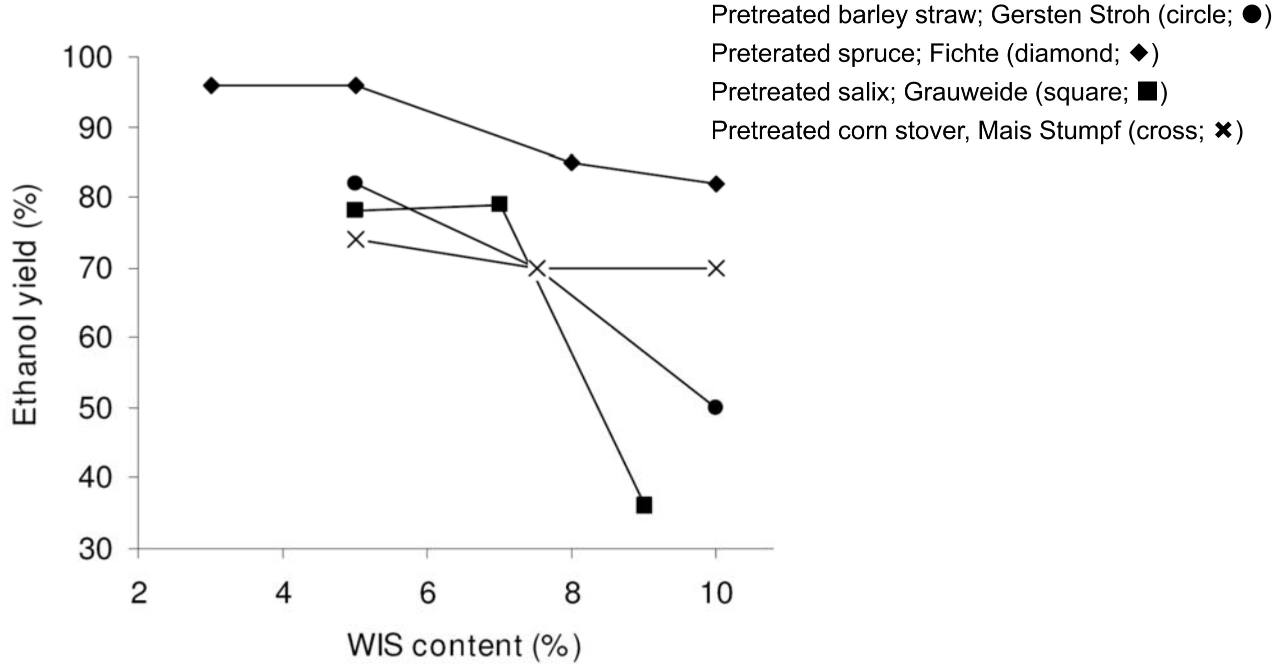


Influence of temperature on the stability of celluclast
Enzyme conc.: 0.9 EGU/mL; pH: 4.8

Celluclast® 1.5 L product sheet

Different factors influencing SSF performance

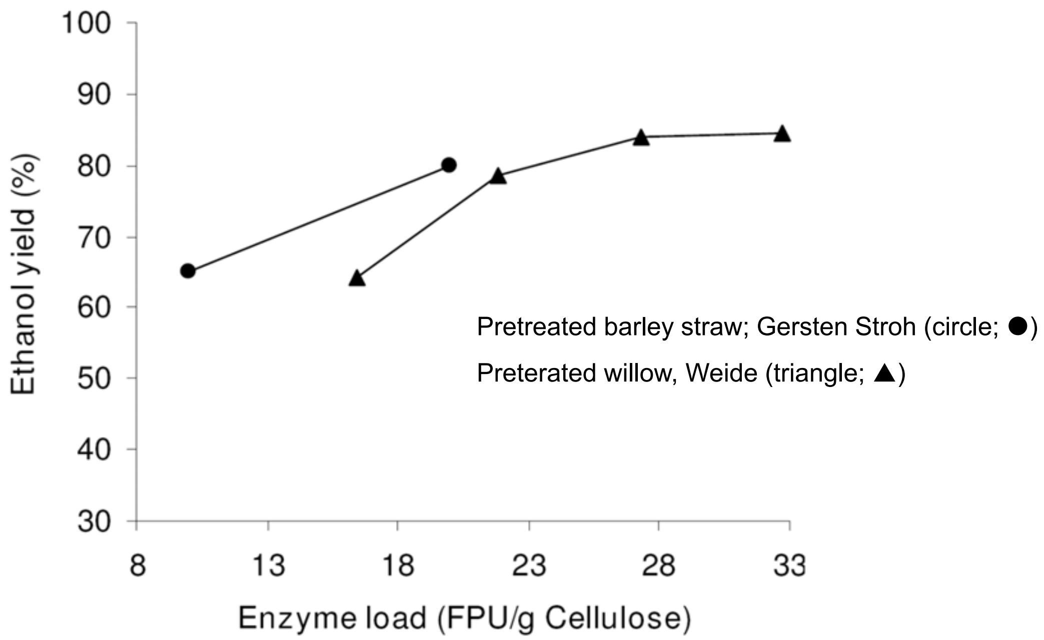
Substrate loading



from Olofsson 2008; *Biotechnology for Biofuels* 2008, 1:7

Different factors influencing SSF performance

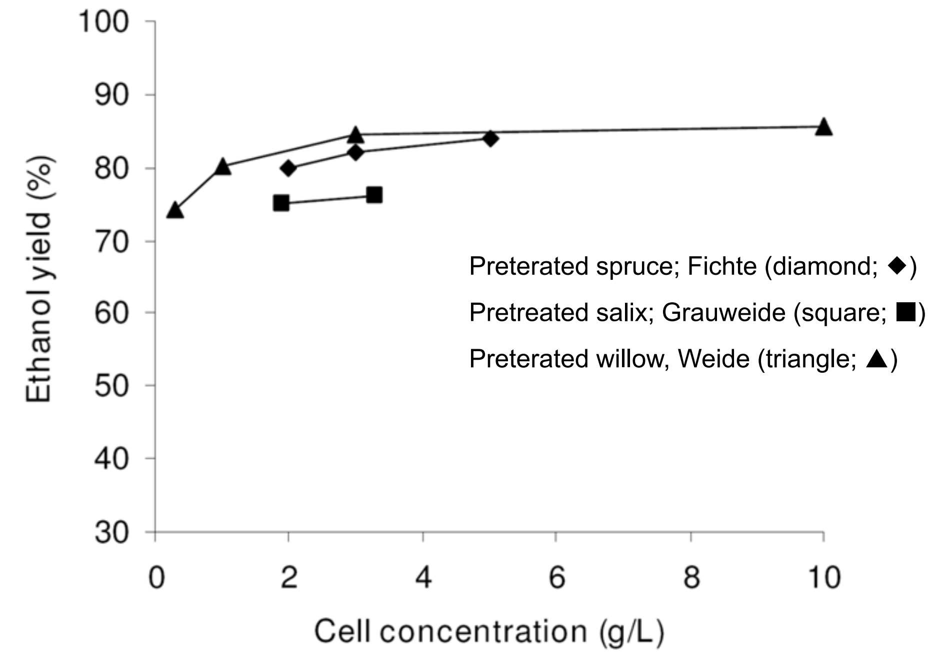
Enzyme loading



from Olofsson 2008; *Biotechnology for Biofuels* 2008, 1:7

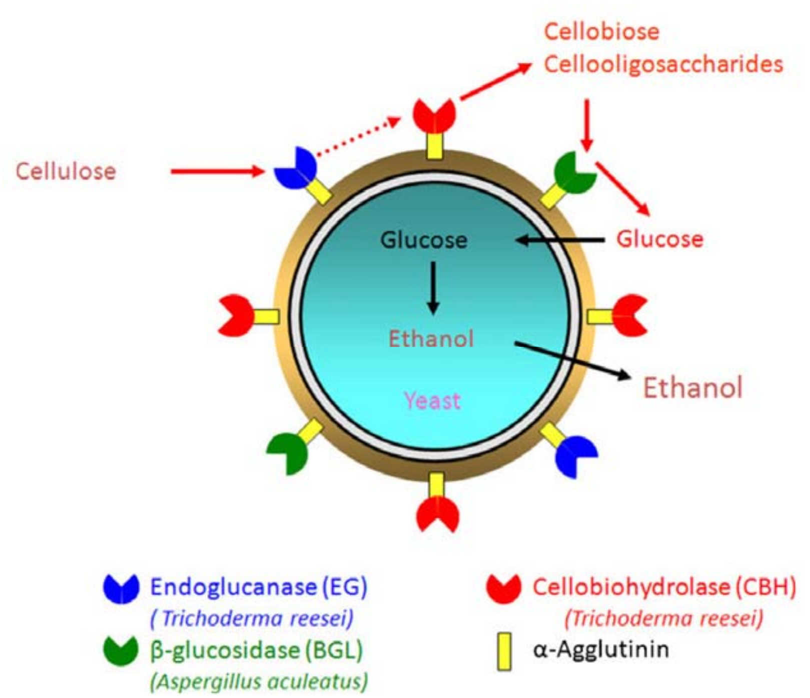
Different factors influencing SSF performance

Cell concentration



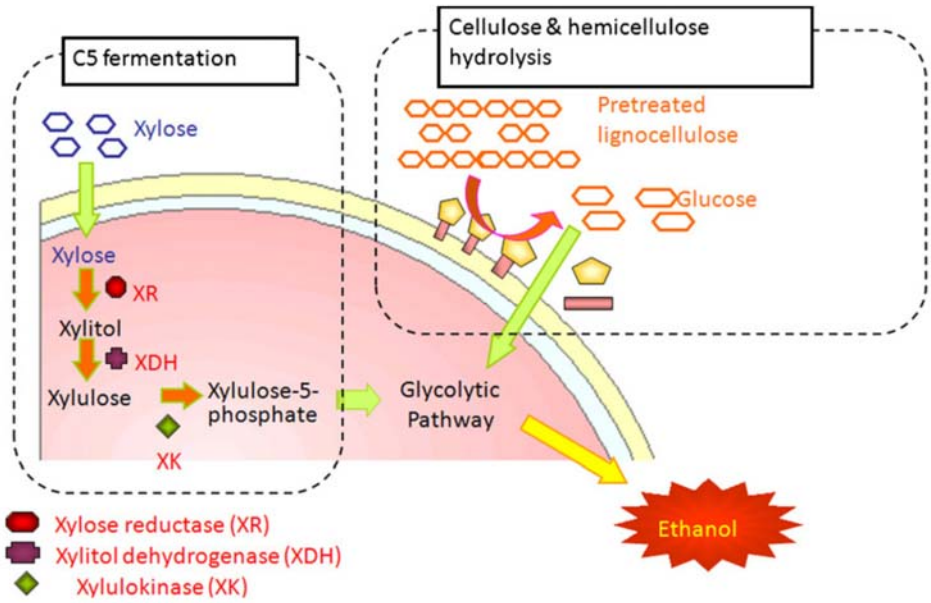
from Olofsson 2008; *Biotechnology for Biofuels* 2008, 1:7

Yeast display for production of ethanol



from Fukuda 2009; Biochemical Engineering Journal 44 (2009) 2–12

Metabolic pathways in pentose assimilating *S. cerevisiae*

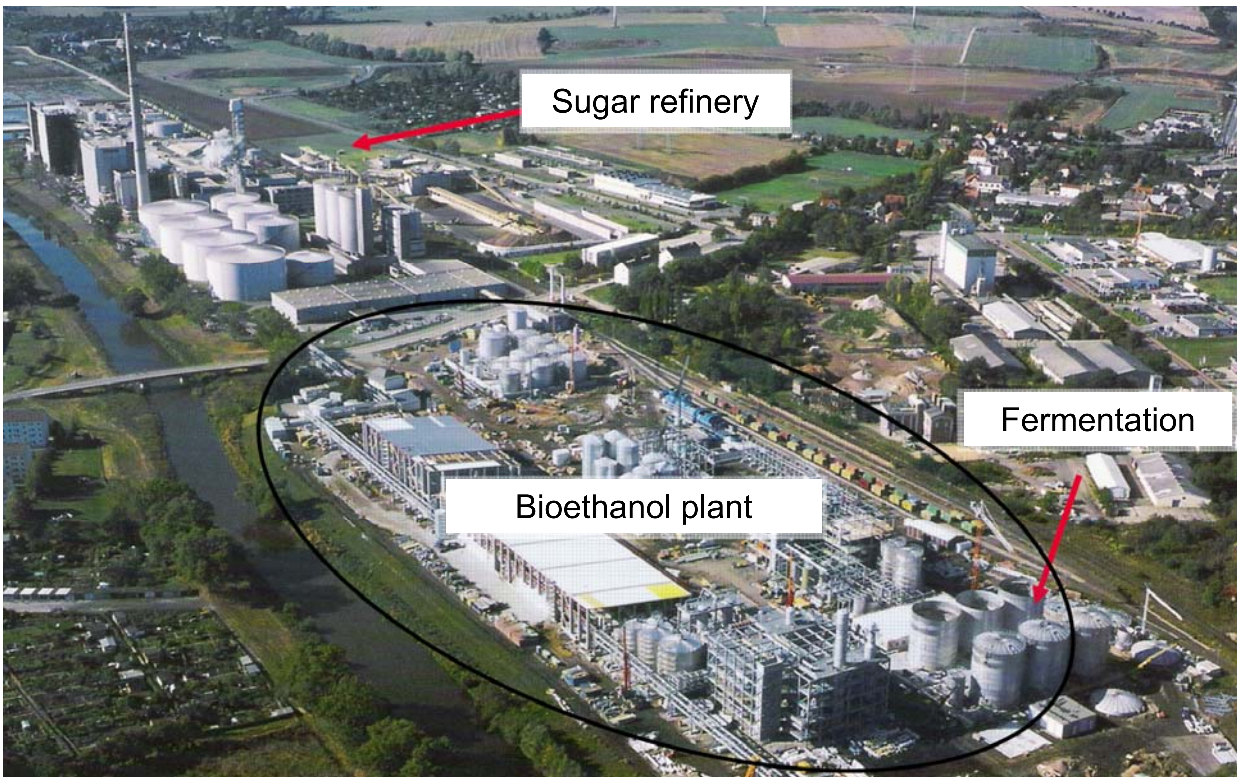


Abbreviations:

XR: xylose reductase; **XDH**: xylose dehydrogenase; **XK**: xylose kinase

from Fukuda 2009; Biochemical Engineering Journal 44 (2009) 2–12

Bioethanol plant Zeitz



Bioethanol plant Zeitz

Start of operation: 2005

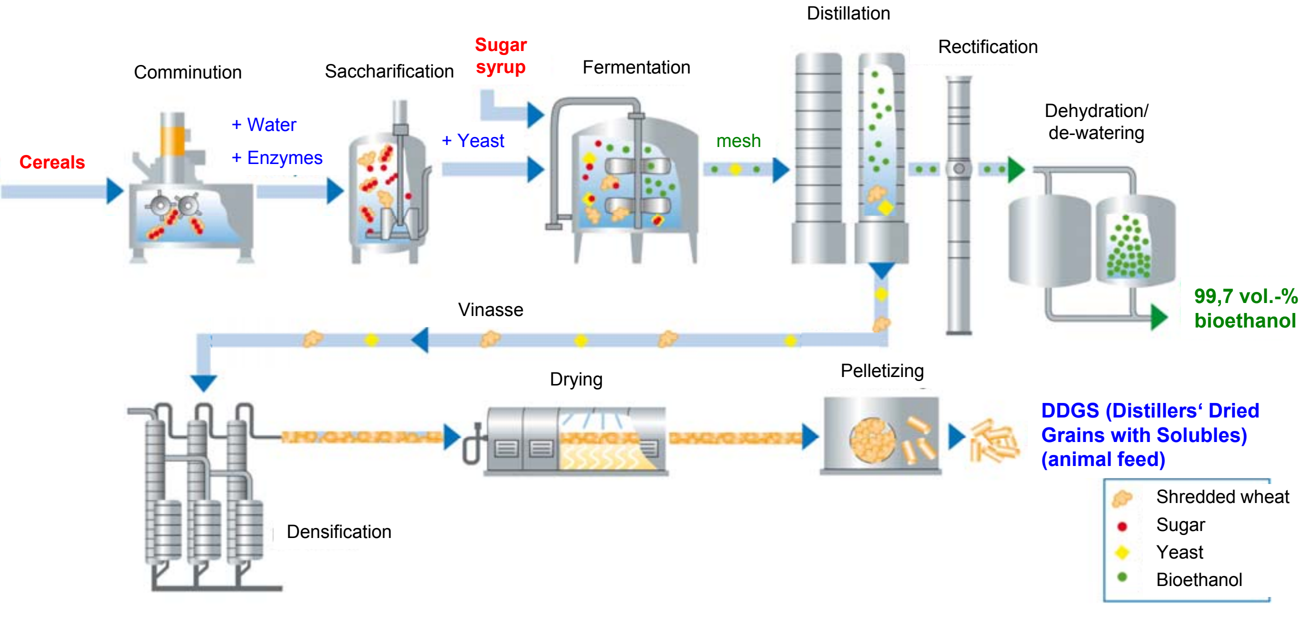
Main raw materials:

- Wheat (700.000 t/a)
- Other cereals
- Sugar beets

Production:

- 260.000 m3/a Bioethanol
- 260.000 t DDGS (Distillers‘ Dried Grains with Solubles) (animal feed)
- 30.000 MWh Strom

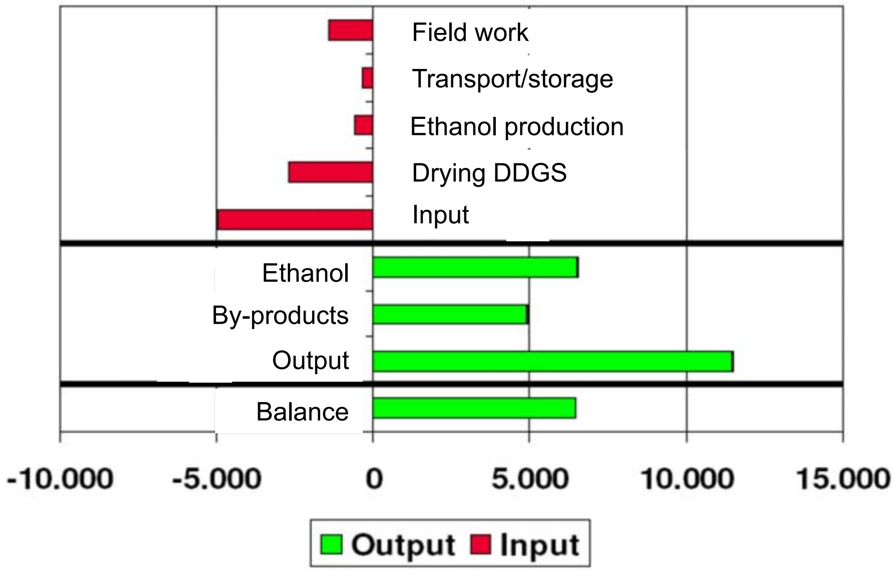
Production schema bioethanol plant Zeitz



Vinasse = Schlempe

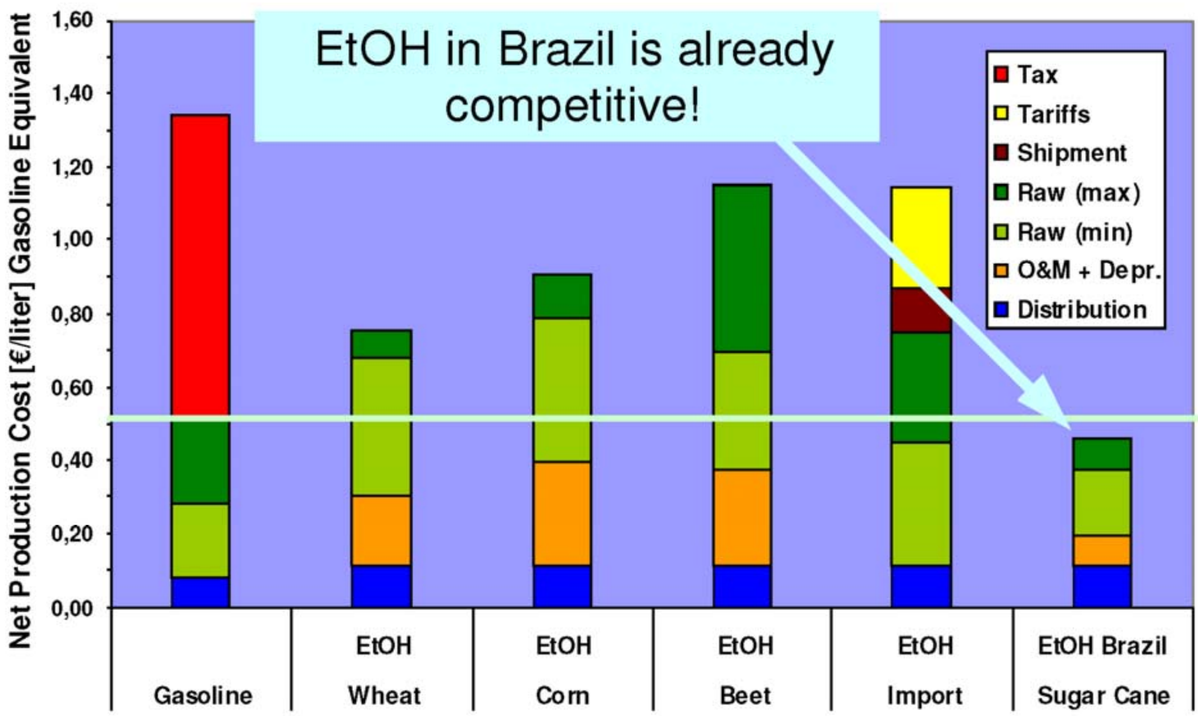
Energy balance of a bioethanol plant in Zeitz

(in kWh/m³ ethanol)



from Dr. Wach, ZAFES, Offstein 2006/05, Südzucker

Economic evaluation of bioethanol production



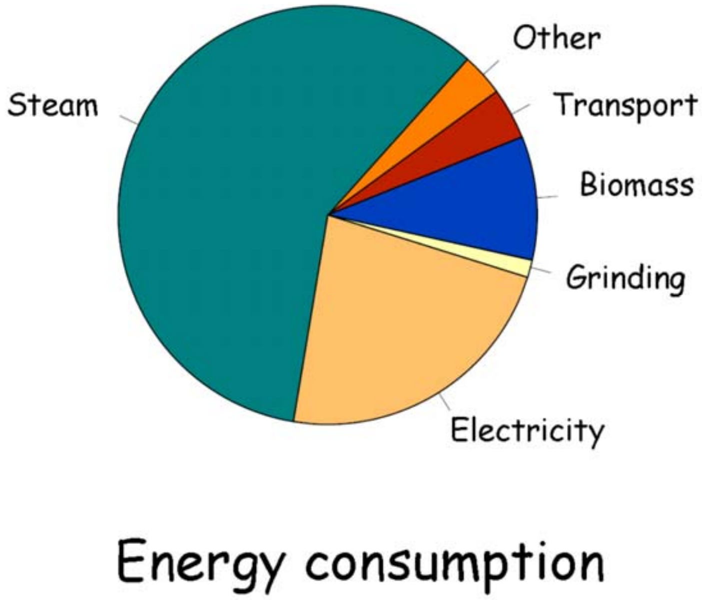
Sources: modelled after BWK Bd. 58 (2006) Nr. 3. S. 53; Luis Carlos Carvalho, Biofuels Perspectives (2005); own calculations

from A. Oberholz, Degussa AG, Marl, March 15th, 2006

Economic evaluation of bioethanol production

The challenge is efficient conversion

- Burning switch grass (10 t/ha) yields 14.6-fold more energy than input to produce*
- But, converting switch grass to ethanol calculated to consume 45% more energy than produced



*Pimentel & Patzek, Nat Res Res 14,65 (2005)

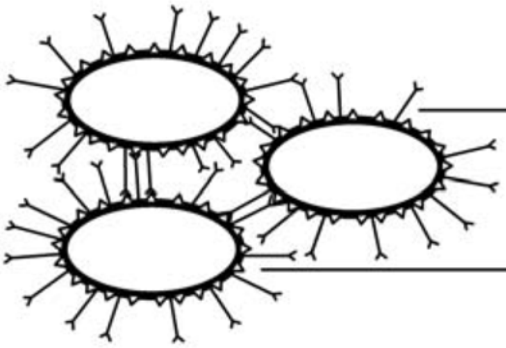
Ethanol production using self-flocculating yeasts

Self-flocculation (self-immobilization) of yeast cells occurs spontaneously

- **No supporting material is needed (more simple and economically and no contamination of later animal feed)**
- **Growth of yeast cells is not significantly affected (ethanol fermentation carried out effectively)**
- **Yeast flocs can be easily purged from the fermentor**
- **Cell separation by sedimentation not centrifugation**

from Bai 2008; Biotechnology Advances 26, p. 89–105

Ethanol production using self-flocculating yeasts



The lectin Model of flocculation

- **Lectin-like proteins (flocculins) stick out of the cell wall of flocculent cells**
- **Selectively bind to cell-wall mannose residues of adjustant cells**
- **Calcium ions are needed to activate the flocculins**

Ethanol production using self-flocculating yeasts

Example of commercial ethanol plant with self-flocculating yeast of BBCA in China

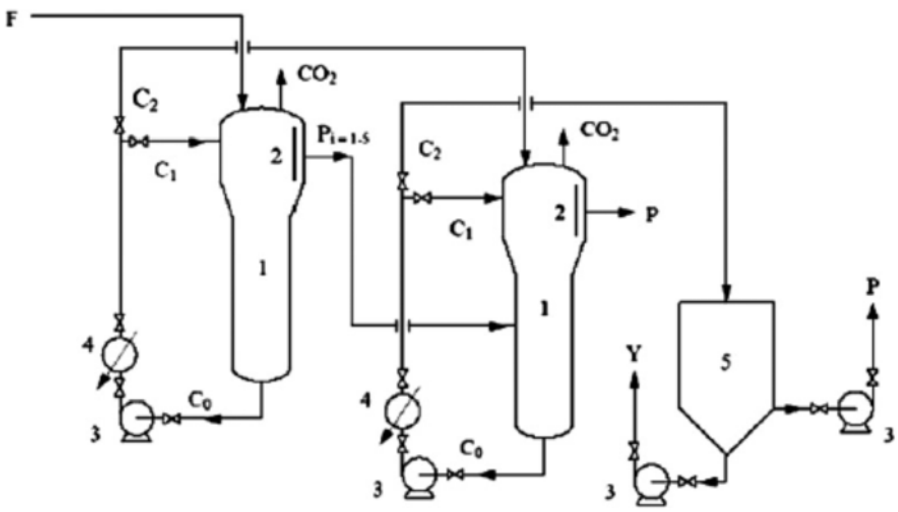
- Annual ethanol production of 200,000 tons
- 6 fermentors with 1000 m^3 working volume in continuous cascade mode (process diagram presented on a later slide)
- Substrate: Corn meal hydrolysate (sugar concentration of 200–220 g/L), is fed into the fermentation system at a dilution rate of 0.05 h^{-1}
- Product: fermented broth with an ethanol concentration of 11– 12% (v/v)

Ethanol production using self-flocculating yeasts

Example of commercial ethanol plant with self-flocculating yeast of BBCA in China

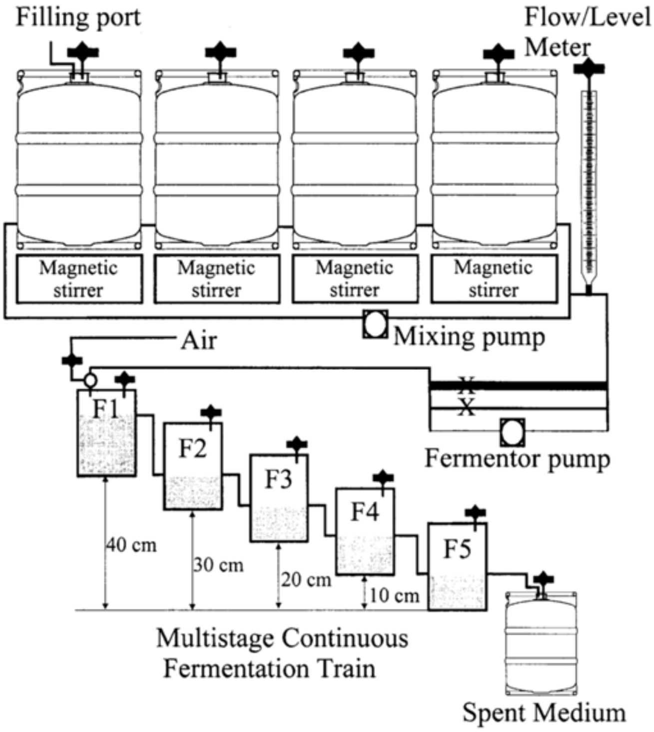
- Yeast flocs are retained within the fermentors by baffles
- Yeast-free broth overflows into the next fermentor or the storage tank for down-stream distillation treatment.
- A small yeast stream bleeding to the next fermentor balances the growth of the yeast cells within the front fermentor.
- The yeast slurry from the last fermentor is transferred to sedimentation tank for yeast flocs separation.

Process diagram for continuous ethanol fermentation with self-flocculating yeast of BBCA



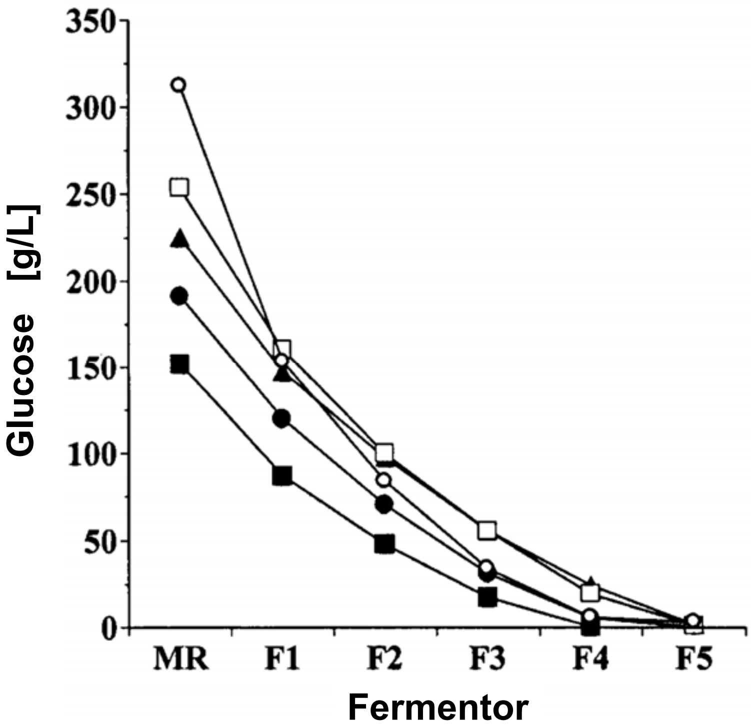
1. fermentors, **2.** baffles, **3.** pumps, **4.** heat exchangers, **5.** sedimentation tank. **F**: substrate stream, **Pi=1–5**: fermented broth, **P**: final product stream, **Y**: yeast paste for post-processing, **C1**: circulating stream, **C2**: yeast bleeding stream. i: number of fermentors in the cascade fermentation system (i=5, last fermentor not included).

Schematic diagram of a multistage continuous fermentation system for ethanol fermentation



from Bayrock 2001, Journal of Industrial Microbiology & Biotechnology 27, 87 –93

Multistage continuous ethanol fermentation system



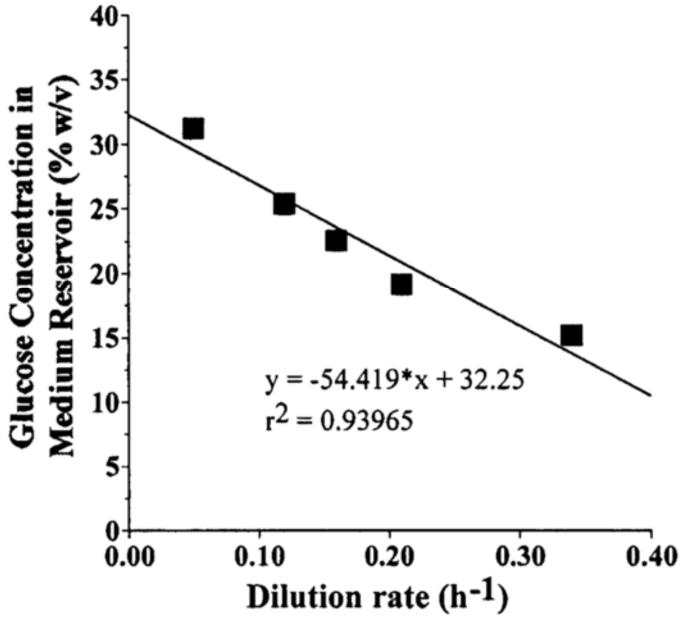
Glucose concentrations in each fermentor at steady state with the medium reservoir containing

- ○ 31.2 % (w/v) glucose (D = 0.05, Flow = 2.33 mL/min)
- □ 25.4 % (w/v) glucose (D = 0.12, Flow = 5.60 mL/min)
- ▲ 22.5 % (w/v) glucose (D = 0.16, Flow = 7.47 mL/min)
- ● 19.1 % (w/v) glucose (D = 0.21, Flow = 9.80 mL/min)
- ■ 15.2 % (w/v) glucose (D = 0.34, Flow = 15.87 mL/min)

MR = medium reservoir
F1–F5 = fermentors 1 –5

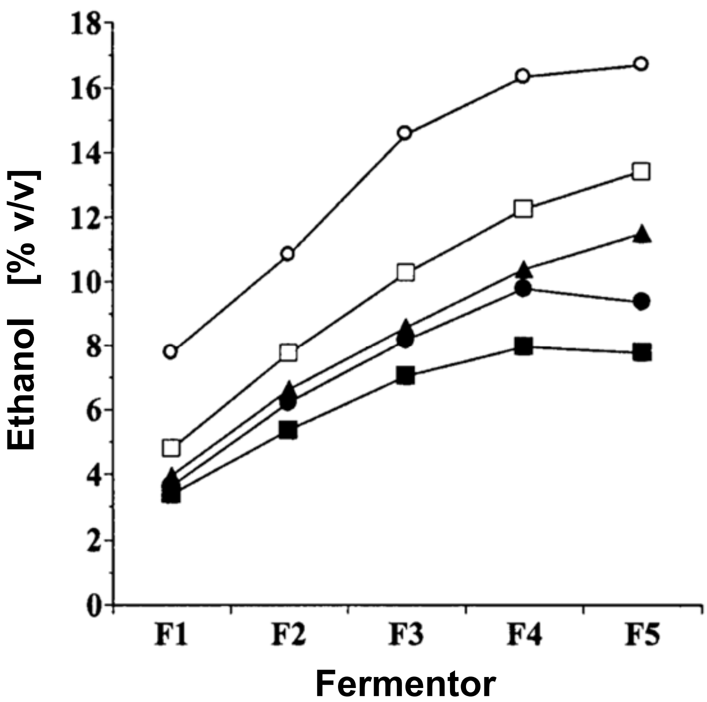
from Bayrock 2001, Journal of Industrial Microbiology & Biotechnology 27, 87 –93

Multistage continuous ethanol fermentation system



Dilution rates required in multistage continuous culture to ensure complete glucose utilization in F5 with increasing medium reservoir glucose concentrations

Multistage continuous ethanol fermentation system



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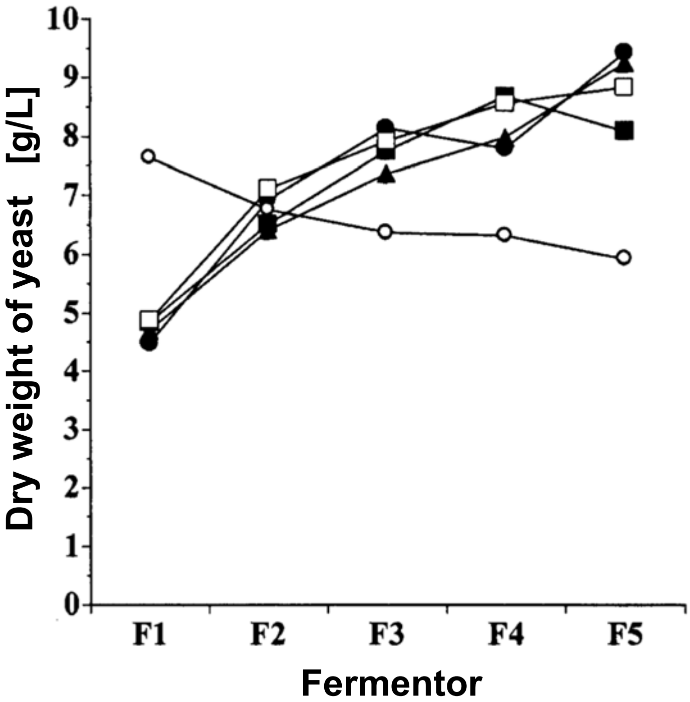
Multistage continuous ethanol fermentation system



Biomass concentrations in each fermentor at steady state with the medium reservoir containing

- ○ 31.2 % (w/v) glucose (D = 0.05, Flow = 2.33 mL/min)
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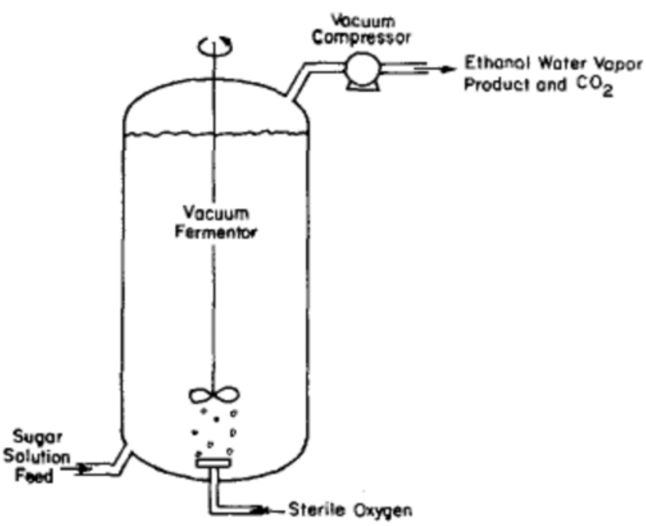
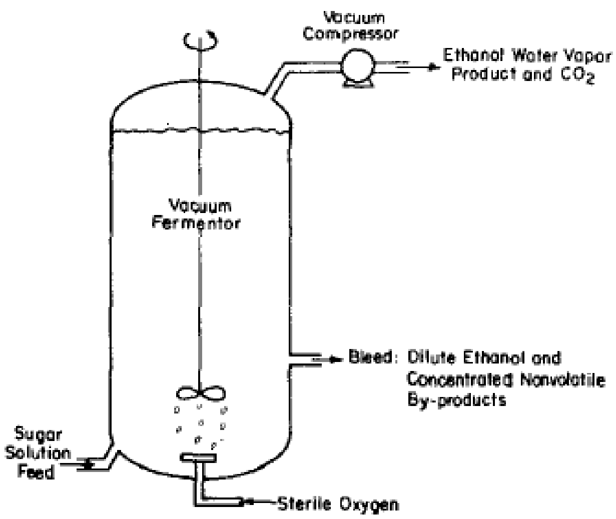
MR = medium reservoir
F1–F5 = fermentors 1 –5

Methods for in-situ recovery of fermentation products

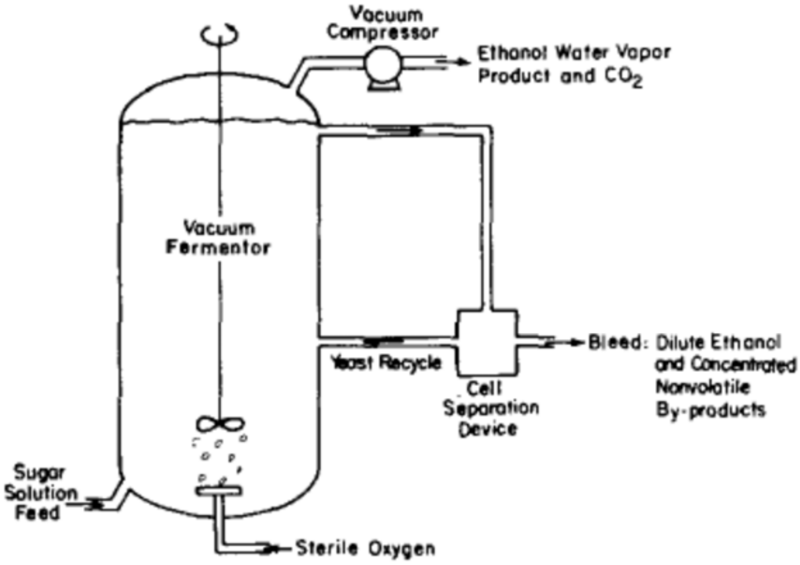


a. Continuous Vacuum Fermentation



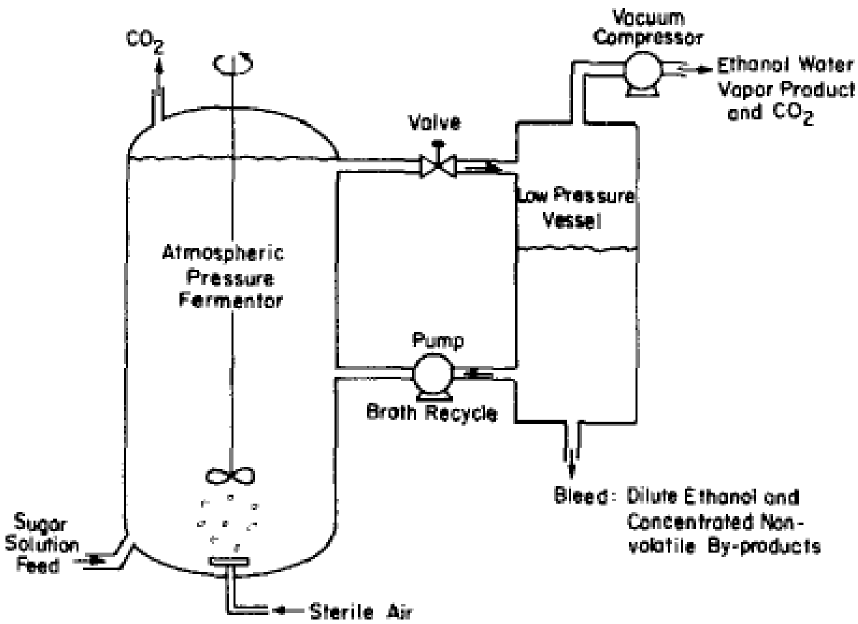
b. Continuous Vacuum Fermentation with Liquid Bleed

Methods for in-situ recovery of fermentation products



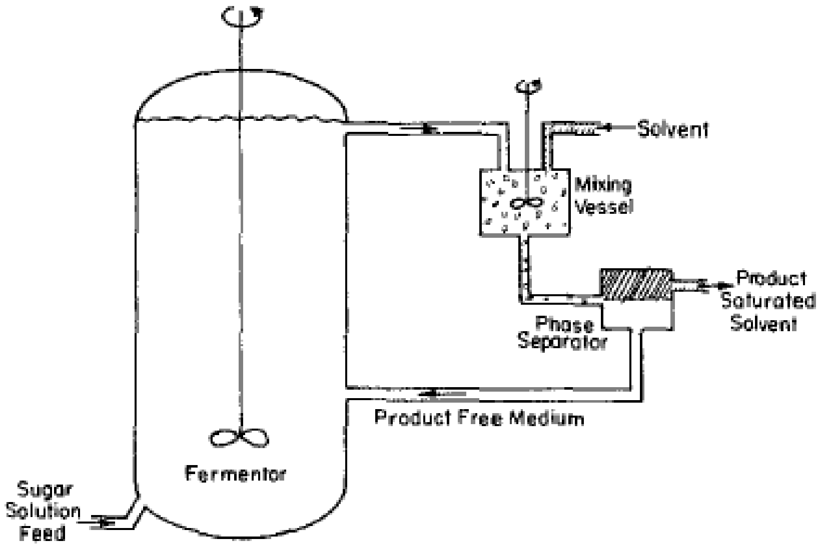
c. Vacuum Fermentation with Yeast Recycle

Methods for in-situ recovery of fermentation products



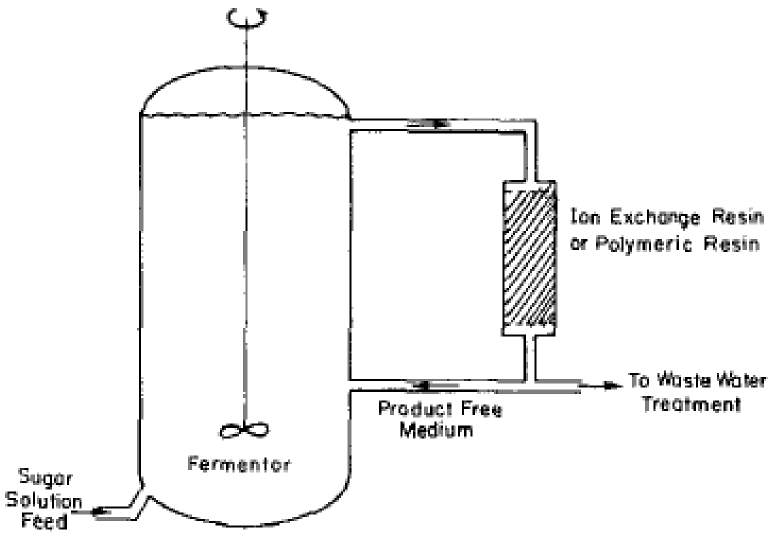
d. Continuous Flash Fermentation

Methods for in-situ recovery of fermentation products



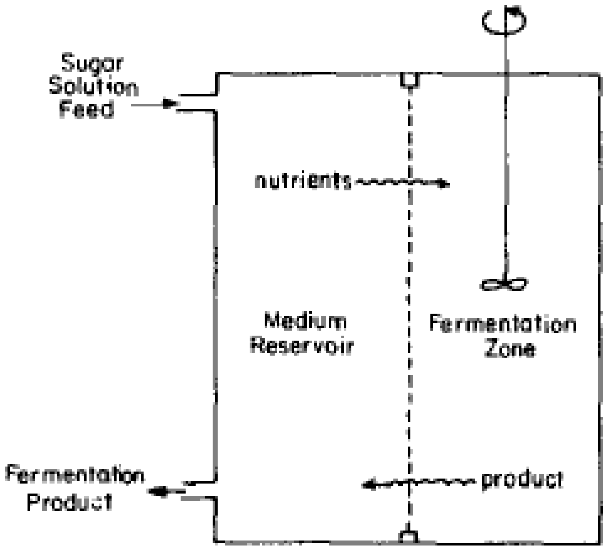
e. Extractive Fermentation

Methods for in-situ recovery of fermentation products



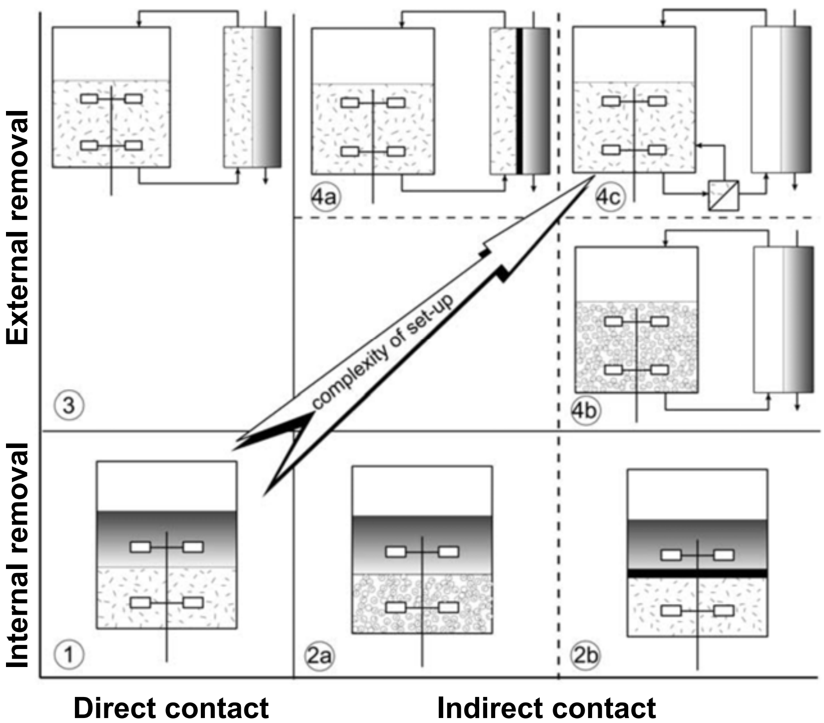
f. Continuous Ion Exchange or Adsorption Fermentation

Methods for in-situ recovery of fermentation products



g. Dialysis Fermentation

Methods for in-situ recovery of fermentation products



1) **Direct contact within reactor**
2a) **Immobilized cells (gel matrix)**
2b) **Internal membrane**
3) **Direct contact external loop**
4a) **External membrane module**
4b) **Immobilized microorganisms in reactor**
4c) **Cell retention before external loop with ultra-filtration**

Visions

- Corn grain ethanol will be displaced by cellulosic fuels (~3-4 fold reduction in land use)
- Sugarcane use will expand to include both sugar and cellulose (~3-4 fold reduction in land use)
- Diesel replacements will be obtained from cellulosic materials rather than vegetable oils (~20-40 fold reduction in temperate acres)
- Ethanol will eventually be displaced by more highly reduced compounds (improved net energy efficiency)
- Synthetic catalysts could be game-changing

"I've always been of the opinion that ethanol is for drinking, not driving."

— Jay Keasling