Table 10. Simultaneous saccharification and fermentation (SSF) of various substrates with and without milling pretreatment (*Trichoderma reesei* QM 9414 cellulase with *Candida brassicae*) [4.51]

Feedstock	Conversion to ethanol			
	Unpretreated		Pretreated	
	24 h	48 h	24 h	48 h
Eucalyptus grandis sawmill waste	0	0	33.44	38.64
Acacia mearnsii sawmill waste	0	0	36.73	45.71
Southern pine sawmill waste	0	0	38.01	42.59
Digester rejects	41.76	54.55	40.31	72.86
Primary clarifier sludge	51.22	66.93	57.49	84.24
Newsprint	34.61	45.40	54.25	60.34
Cardboard	35.12	42.96	53.45	77.93
Air classified sawmill waste	49.58	53.00	59.05	75.13
Cotton linters	10.41	8.74	18.37	16.59
Rice straw	81.49	110.06	57.66	85.47
Rice hulls	45.99	50.19	65.57	96.05
Corn stillage	14.37	16.69	45.57	65.92

The coupled enzymatic hydrolysis of cellulose and simultaneous fermentation of the resulting sugars to ethanol by yeast have been investigated [4.47]–[4.51]. Table 10 shows simultaneous hydrolysis of ball-milled cellulosic substrates by cellulases of *Trichoderma reesei* QM 9414 and conversion of sugars to ethanol by *Candida brassicae*. Use of a thermotolerant yeast and cellulolytic enzymes in a combined saccharification fermentation process is an advantage because the optimum temperature for the hydrolysis of cellulose is 45–50 °C, and cooling problems can be simplified during large-scale fermentation [4.52].

A simultaneous saccharification–fermentation process was also applied in the production of ethanol from starchy materials (cassava and corn). Enzymes from *Aspergillus niger*, *A. awamori*, and *Rhizopus* sp. were used for hydrolysis of starch with simultaneous yeast fermentation [4.53]–[4.55]. Ethanol yields in these processes ranged from 82 to 99% of theoretical. In certain cases ethanol concentration reached a level of 20 vol% in five days [4.53].

The yeast *Schwanniomyces* is able to hydrolyze starch directly and partially ferment it to ethanol [4.56]. Amylolytic systems of *S. castellii* and *S. alluvis* have been reported [4.57]–[4.59]. Economic use of these and other amylolytic species is probably not feasible because of their limited ethanol tolerance [4.56], [4.60].

4.2. Production by Bacteria

In recent years, a number of facultative and obligatory anaerobes have become of increasing interest for the production of ethanol, as shown in Table 11 [4.61].

Thermophilic bacteria grow optimally at 60–70 °C. In the production of fermentation ethanol, their chief advantages over yeasts are

1) cooling costs are lower because less of the heat given off during fermentation has to be removed;
2) less energy is required for stirring the medium because the viscosity of the culture medium decreases with increasing temperature; and
3) the ethanol produced can be continuously separated from the culture medium by using a slightly reduced pressure.

Thermophilic bacteria, however, have a low ethanol tolerance [4.70], [4.71] and, in addition

Table 11. Bacteria of recent interest for the production of ethanol

Organsim	Optimal temperature required for growth, °C	Hexose fermentation	Pentose fermentation	Reference
Facultative anaerobes				
Bacillus macerans	37	+	+	[4.62]
Klebsiella planticola	30	+	+	[4.63]
Obligatory anaerobes				
Zymomonas mobilis	30	+	–	[4.64]
Sarcina ventriculi	37	+	+ *	[4.65], [4.66]
Clostridium saccharolyticum	37	+	+	[4.67]
thermocellum	55–62	+	+	[4.68]
thermosaccharolyticum	58–64	+	+	[4.68]
thermohydrosulfuricum	69	+	+	[4.68]
Thermoanaerobacter ethanolicus	69	+	+	[4.68]
finnii	64–66	+	+	[4.69]
brockii	65–70	+	+ *	[4.68]

* Only arabinose.

to ethanol, produce considerable amounts of lactic and acetic acids during fermentation [4.72], [4.73].

The mesophilic bacterium *Zymomonas mobilis*, isolated at the beginning of the 20th century from palm wine and from the Mexican beverage pulque, appears to be an excellent ethanol producer [4.74], [4.75]. This bacterium has several advantages over yeast (Table 12):

1) its growth rate is approximately double that of yeast;
2) the rate of ethanol production is six to seven times faster than in conventional yeast fermentation, probably because sugar uptake in this small bacterium (size 1–2 μm) is more efficient than in yeast (size 5–10 μm); and
3) the yield of ethanol is ca. 5% higher than in yeast fermentation because less sugar is incorporated into the bacterial cell mass.

Zymomonas mobilis uses the 2-keto-3-deoxy-6-phosphogluconate or Entner–Doudoroff pathway (and not, like yeast, the fructose-1,6-diphosphate or Embden–Meyerhof–Parnas pathway) to metabolize sugar [4.64], pyruvate being formed as an intermediate. Details of these pathways can be found in [4.76] (see also Section 4.1.2 and →Biotechnology, **A 4**, p. 130). The formation of acetaldehyde by decarboxylation of pyruvic acid and its subsequent reduction to ethanol are identical to the reactions in yeast: 2 mol of ethanol and 2 mol of CO_2 are produced per mole of glucose. The energy (ATP) yield of this bacterium, based on the amount of glucose metabolized, is, however, only half that obtained with yeast. Therefore, *Z. mobilis* can synthesize only half as much new cell mass per mole of glucose degraded as yeast does, but this results in a higher ethanol yield.

Table 12. Fermentation properties of the bacterium *Zymomonas mobilis* and of the yeast *Saccharomyces carlsbergensis*

Property	*Zymomonas mobilis*	*Saccharomyces carlsbergensis*
Doubling time, h	2.51	5.64
Ethanol production rate $q_{P/X}$,* g g^{-1} h^{-1}	5.44	0.82
Cell yield $Y_{X/S}$,* g/g	0.028	0.043
Product yield $Y_{P/S}$,* g/g	0.465**	0.460**

* Explanation of symbols:

$$q_{P/X} = \frac{\text{g ethanol formed}}{\text{g cells formed} \cdot \text{h}}$$

$$Y_{X/S} = \frac{\text{g cells formed}}{\text{g substrate used}}$$

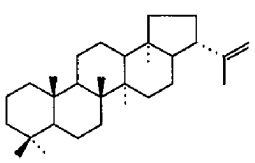
$$Y_{P/S} = \frac{\text{g ethanol formed}}{\text{g substrate used}}$$

** Max. theoretical product yield = 0.511 g/g.

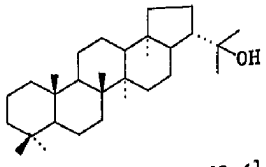
Zymomonas mobilis, unlike yeast, does not require oxygen for growth; this greatly simplifies fermentative ethanol production. In contrast to a number of strictly anaerobic bacteria, *Z. mobilis* is relatively insensitive to oxygen. Indeed, it possesses an electron-transport system similar to the respiratory chain of aerobic organisms [4.77] and can transfer hydrogen, formed in the conversion of glucose to pyruvate, to molecular oxygen. These reducing equivalents are then not available for reduction of acetaldehyde to ethanol; acetaldehyde thus accumulates and inhibits the growth of *Z. mobilis* [4.78].

4.2.1. Ethanol Tolerance

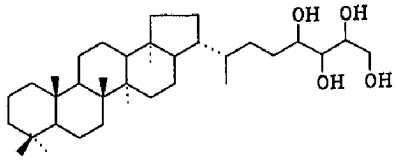
The growth of most bacteria is inhibited by ethanol concentrations of 10–20 g/L. However, both *Z. mobilis* and yeast can tolerate high ethanol concentrations. In fact, alcohol concentrations of 120 g/L have been attained by fermenting sufficiently concentrated sugar solutions with *Z. mobilis* [4.74]. High ethanol concentrations generally destroy the structure and function of the cell membrane [4.79]. Large amounts of pentacyclic triterpenoids (hopanoids), e.g., hopene, diplopterol, and tetrahydroxybacteriohopane, have recently been found in *Z. mobilis*. These substances were first found a few years ago in various mineral oil fractions [4.80], [4.81].



Hopene [*1615-91-4*]
(hop-22(29)-ene diploptene)



Diplopterol [*1721-59-1*]
(hopan-22-ol)



Tetrahydroxybacteriohopane [*51024-98-7*]

The tetrahydroxybacteriohopane content of bacterial cells increases considerably with increasing ethanol concentration (Fig. 10). This indicates that ethanol tolerance is probably linked to hopanoid content. *Zymomonas mobilis* appears to counteract the ethanol-induced changes in fluidity of the membrane by increasing the

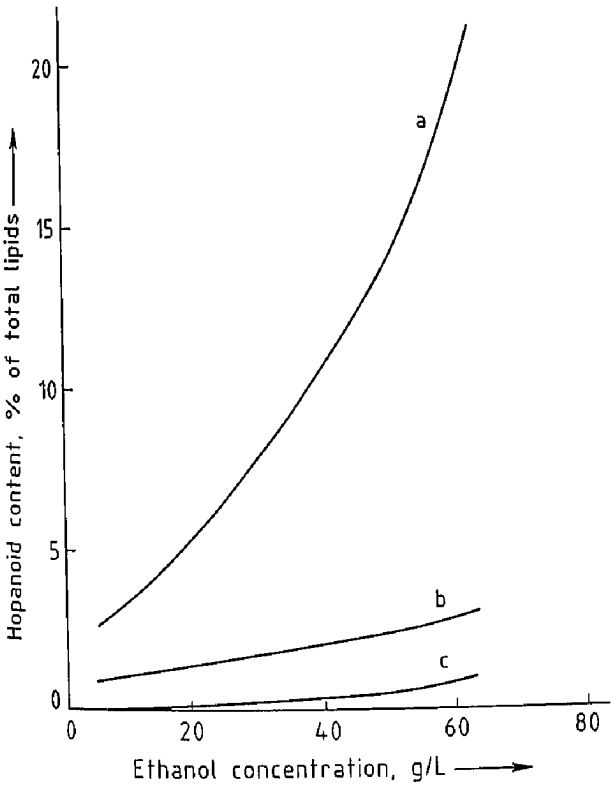


Figure 10. Dependence of the hopanoid content of *Zymomonas mobilis* on ethanol concentration
a) Tetrahydroxybacteriohopane; b) Diplopterol; c) Hopene

biosynthesis and incorporation of hopanoids. The function of hopanoids in bacteria is apparently similar to that of sterols in yeast. They stabilize the membranes, making the microorganism resistant to ethanol [4.82]. Hopanoids are synthesized in bacterial cells under both aerobic and anaerobic conditions, whereas sterol biosynthesis in yeast requires oxygen.

4.2.2. Fermentation of Saccharified Waste Starch

A major consideration in achieving economic ethanol production is the cost of the sugar used as a raw material. For this reason, the fermentation of enzymatically saccharified (hydrolyzed) waste starch by *Z. mobilis* has been carefully investigated [4.83]. Pfeifer & Langen has recently developed a process for isolating glucose syrup from wheat flour (Fig. 11). The wheat flour is first separated into three fractions — pure starch (A-starch), protein (gluten), and a residue (B-starch) [4.84]. The pure A-starch fraction is subsequently converted enzymatically into a liquid sugar product, which is used in the food industry. The residue fraction contains not only starch, but also fibrous material, proteins, and lipids, making it unsuitable for the production of pure glucose syrup. However, after enzymatic liquefaction and saccharification of the waste starch in this fraction, the resulting glucose can be efficiently fermented to ethanol by *Z. mobilis*. Indeed, an ethanol production rate of 3.5–4.5 $g L^{-1} h^{-1}$ and 99% glucose conversion have been achieved in a continuous culture, with dilution rates of 0.06–0.08 h^{-1} and an initial glucose concentration of 120 g/L.

In 1984 Pfeifer & Langen constructed a plant for the continuous production of ethanol by *Z. mobilis* from waste starch fractions. Fermentation carried out in two 70-m^3 fermentors could give a daily production of ca. 10 000 L of 96% alcohol. Ethanol production and the processing of wheat flour are summarized in Figure 11.

After fermentation of glucose to ethanol and its subsequent removal by distillation, stillage is left, which contains the bacterial biomass, fibers, proteins, and fat. These organic substances cannot be degraded by *Z. mobilis*. The wastewater is then fed into a "biogas" plant where a mixed culture of anaerobic bacteria degrades the organic components to biogas, a mixture of methane and CO_2. The methane is used to heat the distillation units.

4.2.3. Fermentation of Pentoses

Yeast and *Z. mobilis* can produce ethanol from glucose, fructose, or sucrose in yields of up to 95%, based on the maximum theoretical yield. However, no yeast or bacterial strains are currently available that can ferment pentoses (e.g., xylose or arabinose) to ethanol as efficiently.

The price of the sugar substrate represents as much as 70% of the total cost of fermentation ethanol. Efficiency could, however, be improved considerably by using both the hexoses and the pentoses of the plant biomass as substrates. A number of facultative and obligatory anaerobes are capable of fermenting pentoses (Table 11, p. 605), but in addition to ethanol and CO_2, they also synthesize large amounts of other fermentation products (e.g., acetic acid, formic acid, lactic acid, acetone, and hydrogen). Metabolic studies show that these bacteria use both the pentose phosphate pathway and the Embden–Meyerhof pathway to metabolize pentoses to pyruvic acid (Fig. 12). In *Z. mobilis*, pyruvic acid is decar-

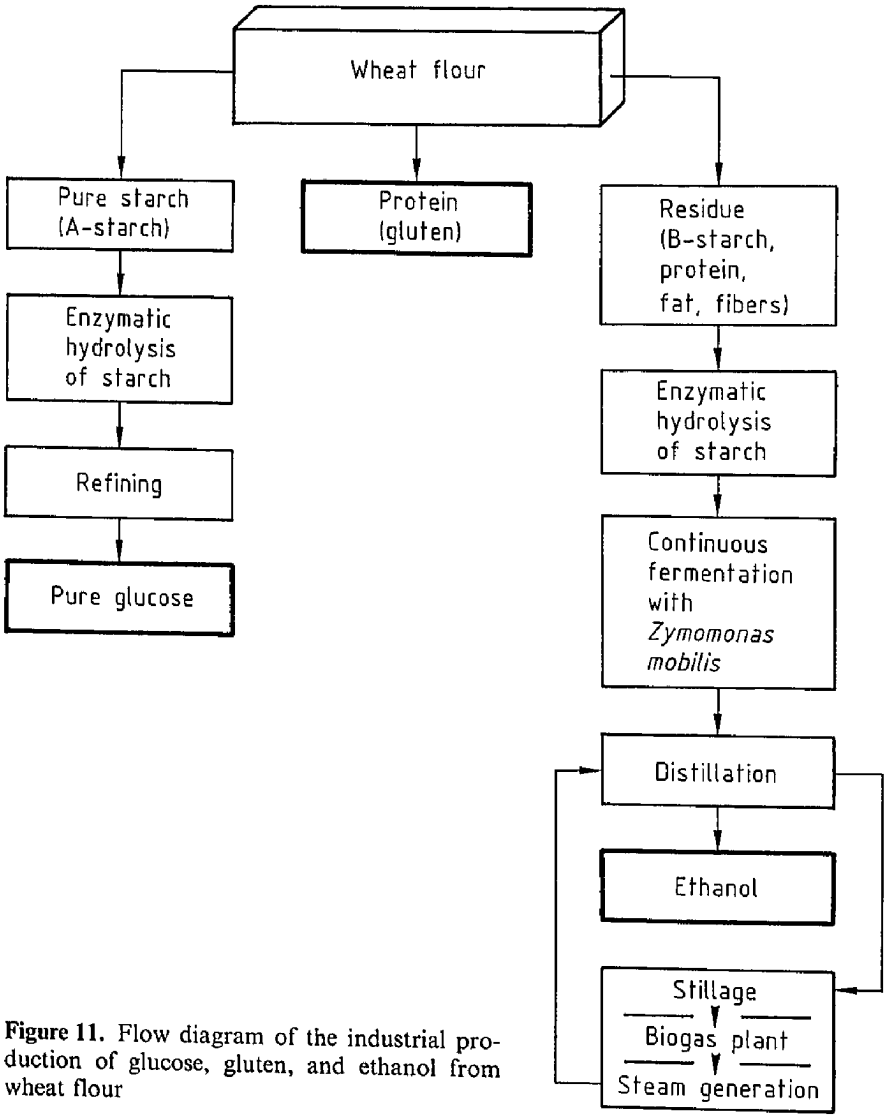


Figure 11. Flow diagram of the industrial production of glucose, gluten, and ethanol from wheat flour

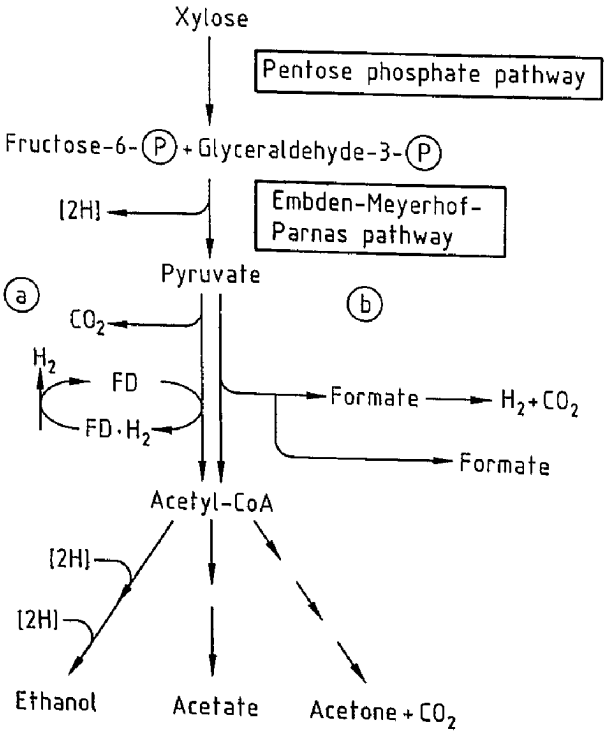


Figure 12. Pentose fermentation pathways in bacteria
a) Pyruvate: ferredoxin oxidoreductase (E.C. 1.2.7.1) [*9082-51-3*] (clostridia); b) Pyruvate formate-lyase (E.C. 2.3.1.54) [*9068-08-0*] (enterobacteria)
FD = ferredoxin; Ⓟ = phosphate

boxylated to acetaldehyde and CO_2 by the key fermentation enzyme pyruvate decarboxylase (E.C. 4.1.1.1) [*9001-04-1*], but facultative and obligatory anaerobic bacteria do not possess this enzyme. Facultative anaerobic bacteria contain pyruvate formate-lyase (formate acetyltransferase E.C. 2.3.1.54) [*9068-08-0*], which cleaves pyruvic acid to formic acid and acetyl-CoA. Some of the formic acid is then converted to hydrogen and CO_2 by formate hydrogen lyase. Strict anaerobes (e.g., clostridia) contain a pyruvic-ferredoxin oxidoreductase (E.C. 1.2.7.1) [*9082-51-3*] and a hydrogenase, which convert pyruvate to acetyl-CoA, hydrogen, and CO_2. The acetyl-CoA formed by these two routes participates in further enzymatic reactions, and some of it is reduced to ethanol. Bacterial pentose metabolism is summarized in Figure 12.

The gene for pyruvate decarboxylase from *Z. mobilis* has been inserted into *Escherichia coli* and *Klebsiella planticola*. In comparison with the wild types, these manipulated strains produce more ethanol and fewer organic acids during the fermentation of sugar [4.63], [4.84], [4.85]. Preliminary results indicate that this technique will prove useful in developing bacteria that can efficiently produce ethanol from pentoses.

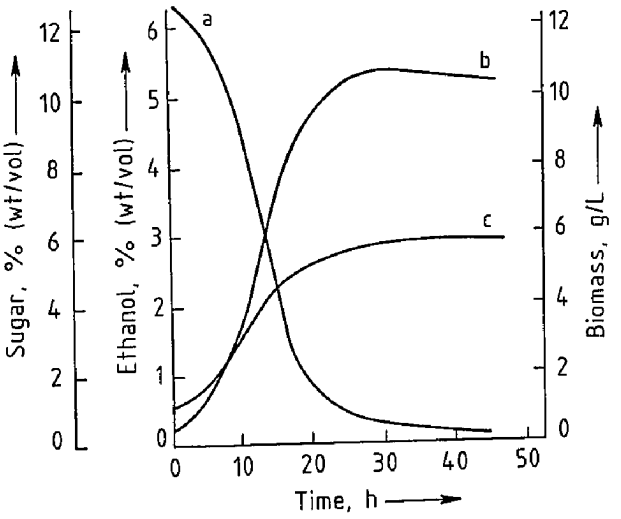


Figure 13. Ethanol production by *Kluyveromyces marxianus* 10606 [4.87]
a) Sugar; b) Ethanol; c) Biomass

Table 13. Effect of initial cell concentration on time of fermentation during batch culture [4.89]

Initial cell concentration, g/L	Time to develop 12 vol% ethanol, h
21.4	7
23.6	6
27.2	5
26.13*	4.5

* With yeast vitamins.

4.3. Fermentation Modes

The two basic modes for production of ethanol by fermentation are the batch and continuous processes. A semicontinuous mode, which is a combination of the two, is also used.

4.3.1. Batch Processes

Most fermentation ethanol is produced by batch processes [4.5], [4.86]. A batchwise process for the fermentation of corn is shown in Figure 24 (p. 619). Typical kinetics in a batch process are shown in Figure 13.

Conversion of sugars in a simple batch system is ca. 75–95% of theoretical, with a final ethanol concentration of 10–16 vol% [4.86], [4.88]. The productivity of simple, conventional batch processes is usually 1.8–2.5 g of ethanol per liter of fermentor volume per hour [4.5].

To increase productivity, a batch process with recycling of the yeast was developed; this is known as the "Melle Boinot" process. Recycling of yeast cells creates a high biomass concentration at the beginning of the process, which reduces the time for the conversion of substrate to ethanol (Table 13) [4.89]. This rapid fermentation also increases productivity. Increasing the initial concentration of yeast cells decreases yield. With a yeast cell concentration of ≥ 21 g/L, no growth was noticed; the existing cells used nutrients for both ethanol production and their own maintenance [4.89].

Under rapid fermentation conditions, a high ethanol concentration is attained quickly and the death rate of yeast cells is quite high. The death rate can be decreased by fermenting at lower temperature and by maintaining the dissolved oxygen concentration in the medium at 10–20% of oxygen saturation [4.39], [4.90]. The tolerance of some yeasts to ethanol is also increased in the presence of yeast vitamins [4.89], [4.91].

Although classic batchwise processes for ethanol production are attractive because of their simplicity, they have many disadvantages such as low productivity, difficulty in automating, long and frequent downtime, and significant labor cost.