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Riassunto

Questo lavoro tratta della produzione di etanolo da diverse biomasse, compreso i materiali lignocellulosici.

Sono riportati cenni al mercato mondiale ed europeo dell'etanolo insieme ad una breve rassegna dei processi impiegati correntemente; si tratta per lo più di tecnologie ormai mature basate sull'utilizzo di piante ad alto contenuto di zucchero o di amido.

Per quanto riguarda la produzione di etanolo da biomasse lignocellulosiche, sono riportati i risultati di un'indagine statistica sulla disponibilità in Europa di questi materiali in termini di coltivazioni dedicate, residui agro-forestali e rifiuti domestici.

È riportata altresì una ricerca bibliografica sui recenti sviluppi dei processi di conversione a etanolo su scala banco e pilota.

Infine, una breve discussione sugli aspetti economici connessi all'utilizzo di materiali lignocellulosici per la produzione di etanolo mostra come questi processi si stiano avvicinando alla completa fattibilità.

Parole chiave: *etanolo, biomassa, lignocellulosa*

Abstact

This 'State of the Art', deals with the ethanol production from current processes based on the use of sugar and starch as feedstock and those under development based on lignocellulosic biomass.

In the first section are reported the commercially available processes together with hints to the newest technologies.

As regard the ethanol production from lignocellulosics, it has been collected data on the biomass availability in Europe as energy crops, industrial crops, agricultural residues and domestic waste.

It is provided a bibliographic study on the technologies and processes under development worldwide for the conversion of lignocellulosics into ethanol.

Finally, a brief discussion on the economics highlights the near term viability of producing ethanol by this way.

Key words: *ethanol, biomass, lignocellulose*

INDEX

INTRODUCTION

There are several ways ethanol can become one of the most important commodities in a close future.
Both pure ethanol and blends of it with gasoline can feed car engines, as it has been successfully proven on commercial scale by the Brazilian experience.
One of its numerous derived products, the ETBE, can be used as octane enhancer and internal oxygen source to improve the combustion efficiency.
Alternatively to the combustion, ethanol can be catalytically reformed to hydrogen that feeds fuel cells on board of an electrical car.
Beside the primary role as energetic product, it is envisaged also an extensive use of ethanol as source of "green" products, *e.g.* the ethyl-lactate that can substitute some CFC as non-halogenated and non-toxic solvent.

A rapid increase of the ethanol demand would lead to a price boosting of the sugar and grains, the feedstocks from which it is currently produced, making more difficult the competition with the fossil fuels.
For example, in Italy the substitution of gasoline with ethanol produced from sugar or grains should be viable only by devoting to this purpose at least 40.000 km^2 of fertile land, corresponding to 26% of the agricultural land (13% of the whole national territory).

These considerations point out that the bioethanol success is connected to the development of new processes able to produce it from alternative feedstocks largely available and cheap, such as residual biomasses and the organic fraction of MSW.

The production of ethanol from cellulose has been investigate since the first decades of the past century, and reached the industrial scale during WWII, when ethanol was produced by acid hydrolysis of wood (the Bergius process).
More recently, several pilot facilities were built after the 70's oil crisis, but they were not scaled up because of the lack of exemption from taxation.

Nowadays, significant advances in the process basics have renewed the interest towards the production of ethanol from lignocellulosics.
The genetic engineering techniques have provided bacterial and yeast strains able to ferment not only glucose, but also the other sugars available in the feedstock.
The improvement of the hydrolytic enzymes is one of the hot topics in this field and relevant progresses are expected in the next few years.

At our best knowledge, several plants are under construction or scheduled in North America and Europe.
Iogen Corp. has built, and nowadays testing, a $25-million plant near Ottawa. The plant is able to treat up to 40 tonnes per day of feedstock, and is the final step before the construction of full-scale, $200-million commercial plants based on the biological (enzymatic) hydrolysis.
The acid hydrolysis will be employed in four plants that are scheduled in USA. The plants will use as feedstocks the lignocellulosic fraction of MSW (New York state, capacity 37 million litres of ethanol per year); bagasse (in Louisiana, 55 million litres per year); rice straw (in California, 55-90 millions litres per year); wood milling waste (Alaska, 22 millions litres per year).
In Europe, Sweden is the most active Country with about ten research groups involved in the basic and applied research and a demonstration plant scheduled for the next year. The plant will be based on the acid process and will use wood waste as feedstock.

These enterprises are the unambiguous gauge that the process is viable both from the technical and economic sides.
Neverthlessr, it seems that a "standard" process has been not yet assessed.
The literature survey shows that both biological (enzymatic) and chemical hydrolysis are under testing, as well different fermentation strategies an byproduct recovery.
In such framework, where *"everyone try to sell its own product"* it is difficult to assess the differences between the processes, the real improvements and the lab to plant scalability.

This 'State of the Art' has been written within the project '*Production of clean hydrogen from fuel cells by reformation of bioethanol'* co-funded by the EU, whose goal is to provide a new way of using biomass as energy source for mobile applications, *via* ethanol.

Although lignocellulosic biomasses are the specific subject of the project, it has also been considered the current ethanol production from sugar and starch.
In the first section are reported the commercially available processes together with hints to the newest technologies.
As regard the ethanol production from lignocellulosic biomass, it has been collected data on the biomass availability in Europe as energy crops, industrial crops, agricultural residues and domestic waste.
It is provided a bibliographic study on the worldwide technologies and processes under development for the conversion of lignocellulosics into ethanol.
Finally, a brief discussion on the economics highlights the near term viability of producing ethanol by this way.

ETHANOL PRODUCTION FROM SUGAR AND STARCH

This section briefly reviews the processes involved in the current production of ethanol from simple sugars or starch. The processes described here relate to the processing of starch from cereals (mostly corn) and sugar from sugar beet or sugar cane, in the North America and Brazil respectively. As the world's major producers, these are also the Countries where the conversion processes involved have been most thoroughly analysed in technical and economic terms.

Innumerable raw materials can be used to produce ethanol, but very few are used for the industrial production, in Tab. 1 the composition of the main feedstocks employed.

Tab. 1 – Typical composition of the main feedstocks used for the ethanol production.

	Sugars	*Fiber*	*Protein*	*Oil/Fat*	*Ash*	*Water*
Sugar Cane	16.5%	3.1%	0.6%	0.1%	0.3%	82.5%
Sugar Beet	20.4%	1.1%	1.1%	0.1%	0.7%	76.6%
Corn	68.3%	2.9%	7.4%	2.8%	0.9%	17.7%
Wheat	69.1-75.4%	1.8-2.3%	9.4-14.0%	1.8-2.5%	1.7%	11.6-14.0%
Oat	67.4%	5.8%	13.1%	6.1%	2.4%	11.0 %

(Source: Center for New Crops & Plants Products. Pardue University)

In Fig. 1 it is reported the geographical distribution around the world of sugar cane and sugar beet crops. As the map shows, the two crops are grown in very different areas, a fact that reflects their different climate requirements.
Sugar cane only grows well in tropical or sub-tropical regions, which explains why it is mostly grown in Brazil, the Caribbean, in parts of India and South Africa.
By contrast, sugar beet grows in temperate or cold regions, so that it is an ideal crop for those parts of Europe where it represents the primary source of edible sugar, though it is also grown in Asia and North America.

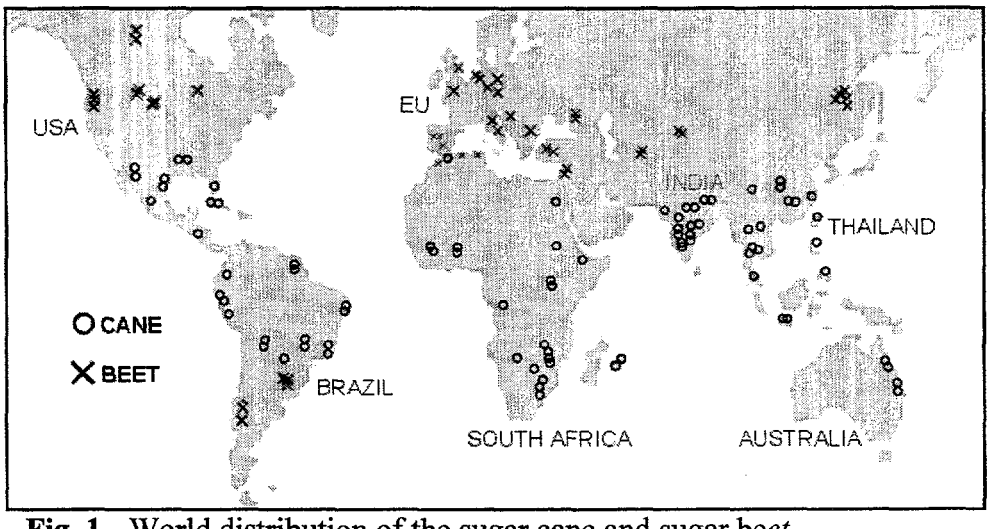


Fig. 1 - World distribution of the sugar cane and sugar be*et*
(Source: Sugar Knowledge International, SKIL)

Whatever the initial biomass, the production of biofuel involves four main steps:

1. Treating the feedstock to obtain a sugar solution
2. Using yeasts or bacteria to convert the sugar into ethanol and CO_2
3. Distilling the ethanol out of the fermentation broth
4. If necessary, dehydrating the ethanol.

PRODUCTION OF ETHANOL FROM SUGAR CANE

Sugar cane contains 12-17% total sugars of which 90% is saccharose and 10% glucose and fructose. Milling extracts roughly 95% of the cane's sugar content (Wheals, A., 1999) leaving behind the solid cane fibre known as "bagasse".

Ethanol is obtained from the cane juice extracted by fermentation but the actual procedures involved will depend on the type of distillery. In Brazil, these come in two types: distilleries that ferment all the cane juice extracted into ethanol and distilleries, which use some of the juice to make sugar.

In the first type, the cane juice is heated to 110°C to reduce the risk of bacterial contamination, then decanted (in some cases after concentration by evaporation) and then fermented.
In distilleries that produce both sugar and ethanol, the crystals formed by the concentration process are centrifuged out, leaving behind the very thick syrup known as molasses, which contains up to 65% w/w sugars and is the part destined to fermentation.
In both cases, the sugar content has to be adjusted to 14-18% for the ferments to work at maximum efficiency. At that point the solution contains sufficient organic and mineral nutrients to ensure fermentation by *Saccharomyces c.*, the yeast most commonly used for the conversion of saccharose, glucose and fructose into ethanol. Fermentation generally takes place at a temperature of 33-35°C and a cell density of 8-17% v/v and is stopped when the ethanol content of the liquor reaches around 10%, since beyond that point the ethanol will start to kill off the yeast.
In Brazil, about 70% of distilleries adopt a discontinuous fermentation process (Wheals, A., 1999).
Under this system, at the end of the fermentation process, the liquor moves on to the distillation phase during which hydrous ethanol (95.5% v/v ethanol- 4.5% v/v water, azeotropic solution) is extracted. From this, higher-grade ethanol (anhydrous ethanol) can be obtained either by dehydration on molecular sieves or by distillation involving benzene or cyclohexane (azeotropic distillation).

The distillation process produces both ethanol and a watery solution called "stillage" or "vinasse". Distillation produces 10-15 times as much vinasse as ethanol. In sugar cane distilleries that vinasse is generally used to irrigate and fertilise the cane fields.
The flow-chart in Fig. 2 depicts the simultaneous sugar ethanol production process. The flow-chart for *solo* ethanol production is naturally similar but without the sugar-producing lines.

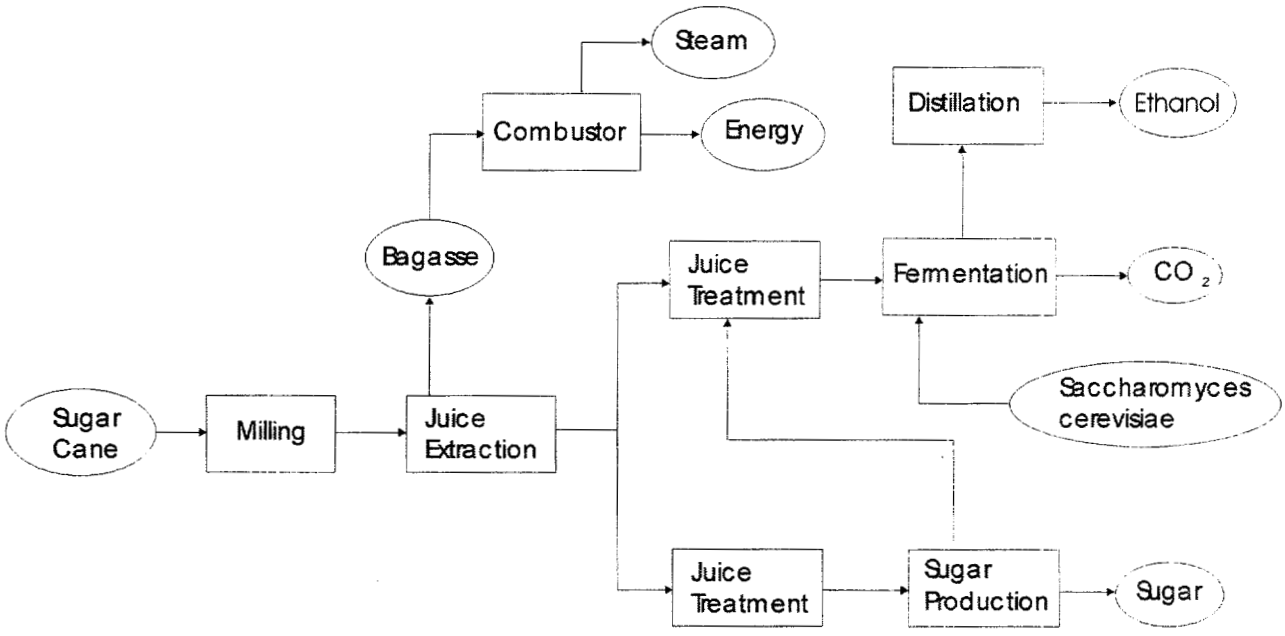


Fig. 2 - Flow chart of the sugar cane processing relative to a plant for both ethanol and sugar production.

PRODUCTION OF ETHANOL FROM SUGAR BEET

Just like sugar cane, sugar beet can produce ethanol *via* the fermentation of its fermented juice and distilleries may produce ethanol alone or both sugar and ethanol (Fig. 3).
The sugar is extracted by diffusion from the pre-sliced beet During this stage, the beet slices are placed in contact with the extraction *medium* (water or beet juice extracted later in the procedure) and held at a temperature of 70-80°C. Temperature is a crucial extraction parameter, since it has to be high enough to break down the proteins in the cell walls that contain the sugar in order for diffusion towards the extraction *medium* to take place.
Once the diffusion process is completed, the drained beet pulp is dried for sale as animal feed or sold to the chemicals and pharmaceuticals industries for use in the manufacture of chemicals like citric acid and its esters. Meanwhile, the juice goes on to further processing.

In mixed ethanol/sugar distilleries the molasses is also sent for processing.

The fermentation process may employ yeasts like *Saccharomyces c.* or bacteria like *Zymomonas mobilis.* However, bacteria have only been used at batch level, so far.
The interest in the use of bacteria derives from the fact that they convert glucose into ethanol more efficiently than yeasts. Laboratory trials have also shown that *Zymomonas mobilis* can usefully be employed for the production of several co-products with a real market value, *e.g.* fructose, sorbitol, gluconic acid, and levan (an interesting substitute for blood plasma), (Linde, 1998).

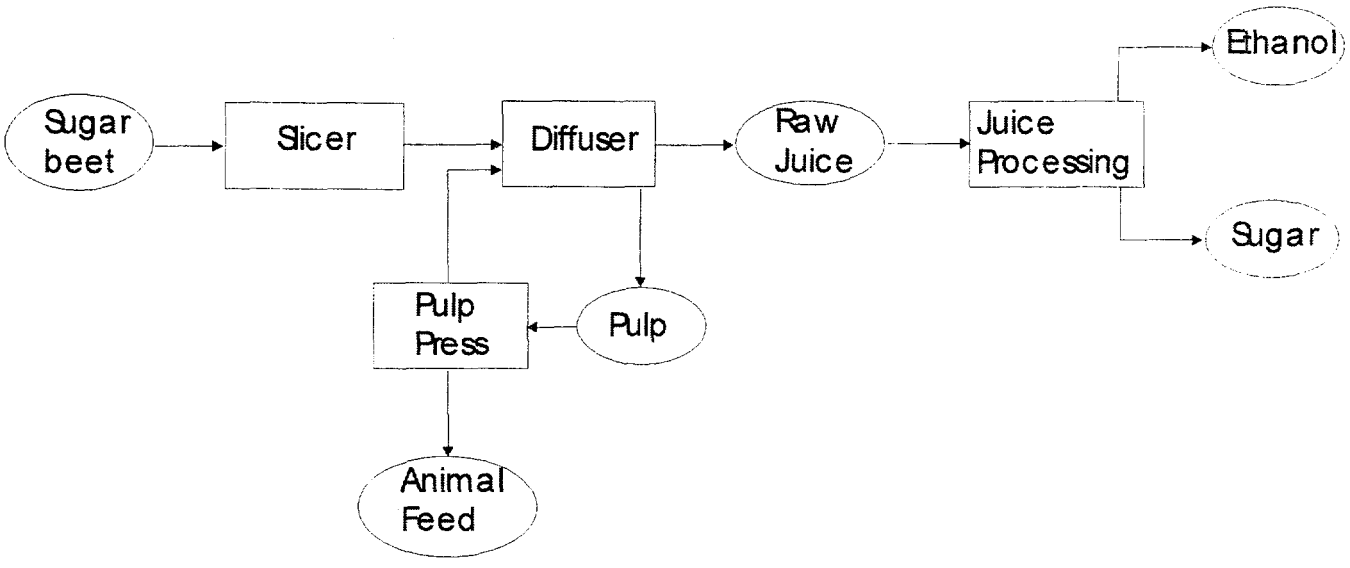


Fig. 3 - Flow chart of sugar beet processing relative to a plant for both ethanol and sugar production.

PRODUCTION OF ETHANOL FROM STARCH

Starch is a natural polymer present in many tubers and cereals. Its constituent monomers are glucose molecules held together by bonds between an oxygen atom on one molecule and a carbon atom on its neighbour. Those bonds are called glycosides and may be α or β type depending on their stereoisomerism to anomeric carbon.
In the plants and seeds that contain it, starch takes the form of granules each containing two main constituents: amylose (about 20%) and amylopectin (about 80%), which are true glucose polymers (α-type). Both polymers are easily hydrolysed and fermented into ethanol, Fig. 4 and 5 show their structures.

The world's most important starch-based ethanol comes from cereals, primarily corn and wheat. Various procedures are employed for the conversion of cereal starches into ethanol, of which some are described here. The first two are the most commonly used at industrial scale, while the others are still in the experimental stage, all of them involving additions to the processes used in the first two, for the purpose of cutting the cost of producing ethanol from starch. In fact the possibility of obtaining co-products is of major significance when the ethanol is distilled from cereal feedstocks other than corn, such as wheat, barley or oat, all of which generally cost more than corn (Tibelius, 1996).

Wet Milling
The process used in producing ethanol from starch is, with a few minor adjustments, the standard procedure long used by the food-starch industry.
Though it demands more power, costs more and yields less ethanol, the wet milling process is industrially preferable because it delivers purer starch and higher value co-products (Tab. 2).
The wet milling of corn produces not just ethanol but several other products: corn oil, gluten feed, gluten meal and CSL (Corn Steep Liquor) each of which has a market value. Corn oil is the most highly prized and is for human consumption; gluten feed and gluten meal both go for animal feed and differ in their protein content (feed 20%, meal 60%). CSL is about 50% protein and for this reason is usually marketed mixed with gluten feed unless it is used in fermentation

processes as an additive to the synthetic growth media used in cultivating micro-organisms (Girelli, 1986).

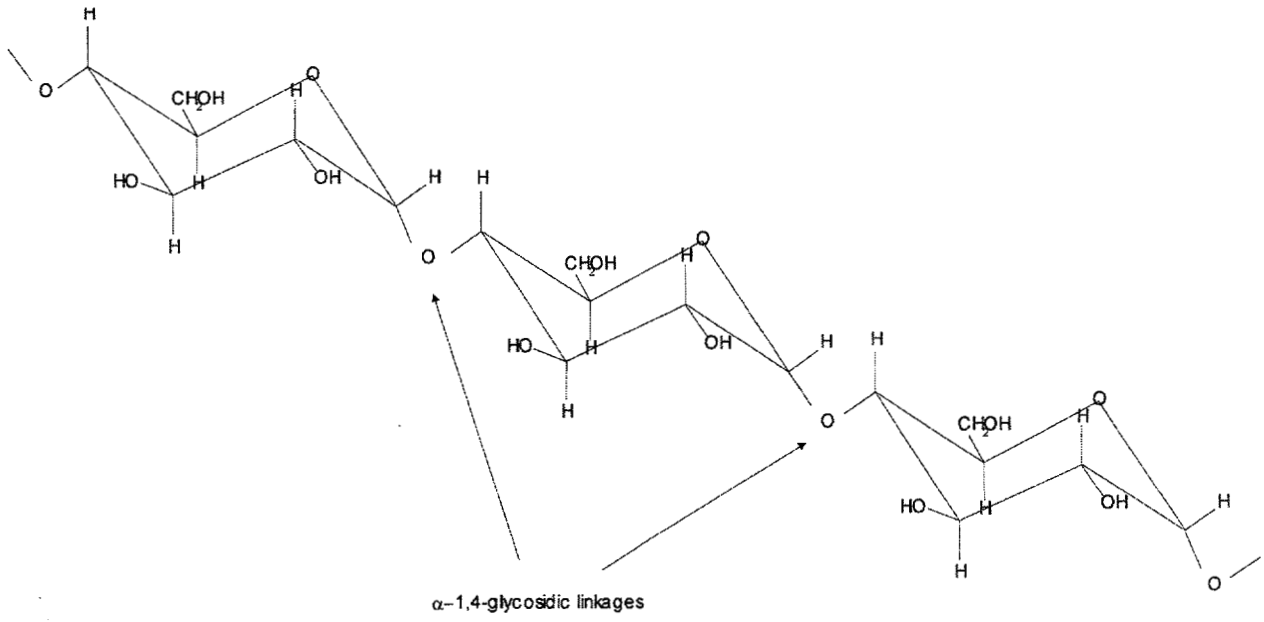


Fig. 4 - Structure of Amylose, the linear polymeric molecule of glucose

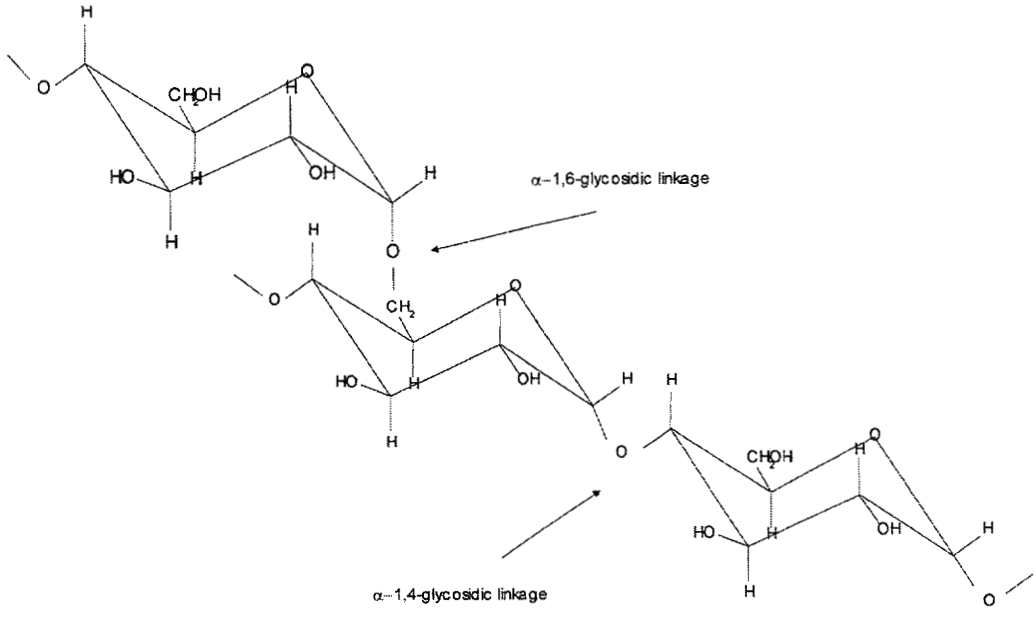


Fig.5 - Structure of Amylopectin, the branched polymeric molecule of glucose

Tab. 2 - Composition of Corn Wet Milling Product (% wt on dry basis).

	Starch	*Protein*	*Other*[a)]	*Moisture*[b)]
Corn Steep Liquor	0	45	55	35-50
Gluten Meal	20	60	20	10
Gluten Feed	10	20	70	10

a) Oil, fibre, ash, salts, sugars and B-group vitamins; b) in the wet products. (Source: C.E. Wyman, 1996)

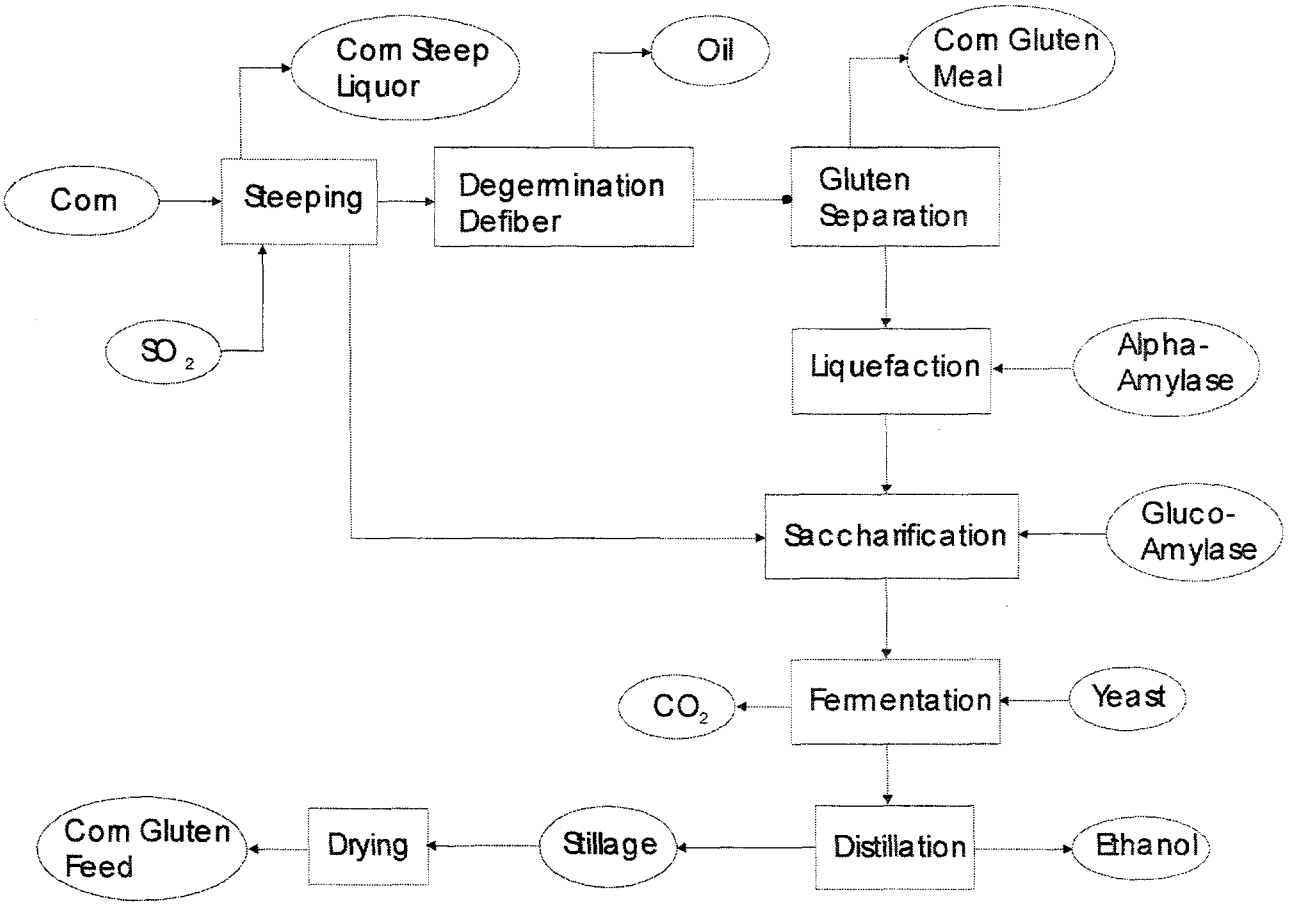
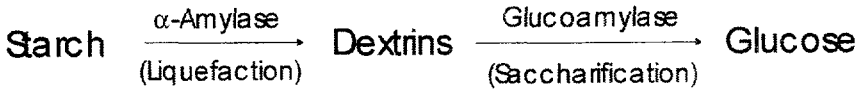


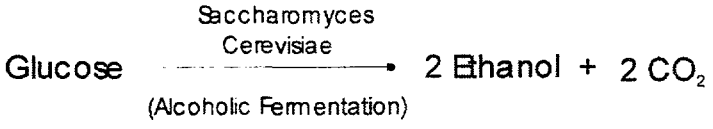
Fig. 6 - Flow chart of wet milling process relative to a plant for ethanol production from corn.

The chart in Fig. 6 shows the flows in the wet milling process.
First the corn is steeped in an 0.1%-0.2% SO_2 water solution for 24-48 hours at around 50°C. By the end of this treatment the corn grains are soft enough for easy milling.
Oil, protein and a starch-rich fraction are then separated out of the resulting meal. It is the starch that then goes off for conversion into ethanol.
Meanwhile, part of the steep water is concentrated into about 50% of its volume in dry matter to become CSL, while the remainder is dispatched to the saccharification plant where it is used to adjust the pH to a slightly acid 4-4.5 and also supplies nutrients for the subsequent fermentation

phase, as well as being used to dilute the ethanol in the fermentation broth to a level tolerated by the yeast.
After extraction the starch is gelatinised by treatment at low and high temperatures. The gel temperature is then raised to around 70°C and NaOH is added to achieve a pH of 5.5-6.2 prior to the addition of α-amylase. $CaCl_2$ is also added to stabilise the enzyme.
The α-amylase breaks down the starch into smaller dextrin molecules. This process, which reduces the viscosity of the starch, is known as *liquefaction.*
After liquefaction, the paste moves on to the *saccharification* phase, during which the action of glucoamylase hydrolyses the dextrins into glucose. Complete saccharification takes 45-72 hours.



This completes the preparation phase so that the glucose solution can be *fermented* into ethanol. The glucose temperature is first lowered to 35° before yeast, generally *Saccharomyces c.*, is added, starting a process of anaerobic fermentation that breaks the glucose down into ethanol and carbon dioxide.



Both batch and continuous fermentation systems are employed.
Once the fermentation liquor reaches an ethanol concentration of around 8-10% v/v beyond which the yeast cannot survive, it is distilled to produce, usually, 95% pure ethanol.
Purer ethanol can be obtained by distilling the ternary mixture obtained by adding benzene or cyclohexane or by drying on molecular sieves.
In some distilleries, beside ethanol also CO_2 is recovered, purified, compressed and sold for a variety of uses (as an additive to fizzy drinks, dry ice or fire extinguishers).

Some distilleries use slightly modified forms of the sequence illustrated above.
There are two major modifications. One involves simultaneous saccharification/fermentation. The second allows for the retrieval of the yeast, which is centrifuged out before the liquor is distilled. This means that the yeast can be reutilised in the fermentation tank, which is cheaper than buying new yeast. However, recycled yeast does require the addition of antibiotics in order to reduce the risk of bacterial contamination.

The wet milling process described above is what the industry calls the "standard process". However, there is a simplified version known as Modified Wet Milling, which eliminates the gluten recovery stage. This demands less equipment and hence a lower capital investment.
In these distilleries, the solution that remains after fermentation is called "stillage". Available in 10-15 times the volume of the ethanol, this stillage can be subjected to further fractionization, centrifuged into a solid part (distillers' grain) which is rich in proteins, fibre and fats and a liquid part called "thin stillage" rich in yeast, sugars and tiny corn particles ("distillers" solubles) which can be retrieved by evaporation. The two dry products (distillers' dried grain or DDG and distillers' dried soluble or DDS) can be sold as they are or as a mixture (distillers'

dried grain with soluble or DDGS). Either way they have a nutritional value and are typically employed as animal feed.
In the simplified distilleries, the thin stillage is not all processed, some of it being used by the distillery in back set fermentation for the purpose of adding nutrients to and optimising the pH of the fermentation liquor.

Dry Milling
This process involves no preliminary treatment of the corn, which is simply milled, mixed with water and heated before going on to the hydrolysing, fermentation and distilling phases. The conversion of the starch into ethanol takes place exactly as in wet milling (Fig. 7).
Since dry milling does not break down the cereal into its various components, the stillage that can be retrieved at the end of the distillation process that extracts ethanol from the fermentation liquor is very rich in valuable nutrients (proteins, fibre, fats, sugars) much richer, in fact, than the stillage obtained in modified wet milling (Maiorella *et al.,* 1983). Therefore it is processed at the end of the distillation phase as in modified wet milling.

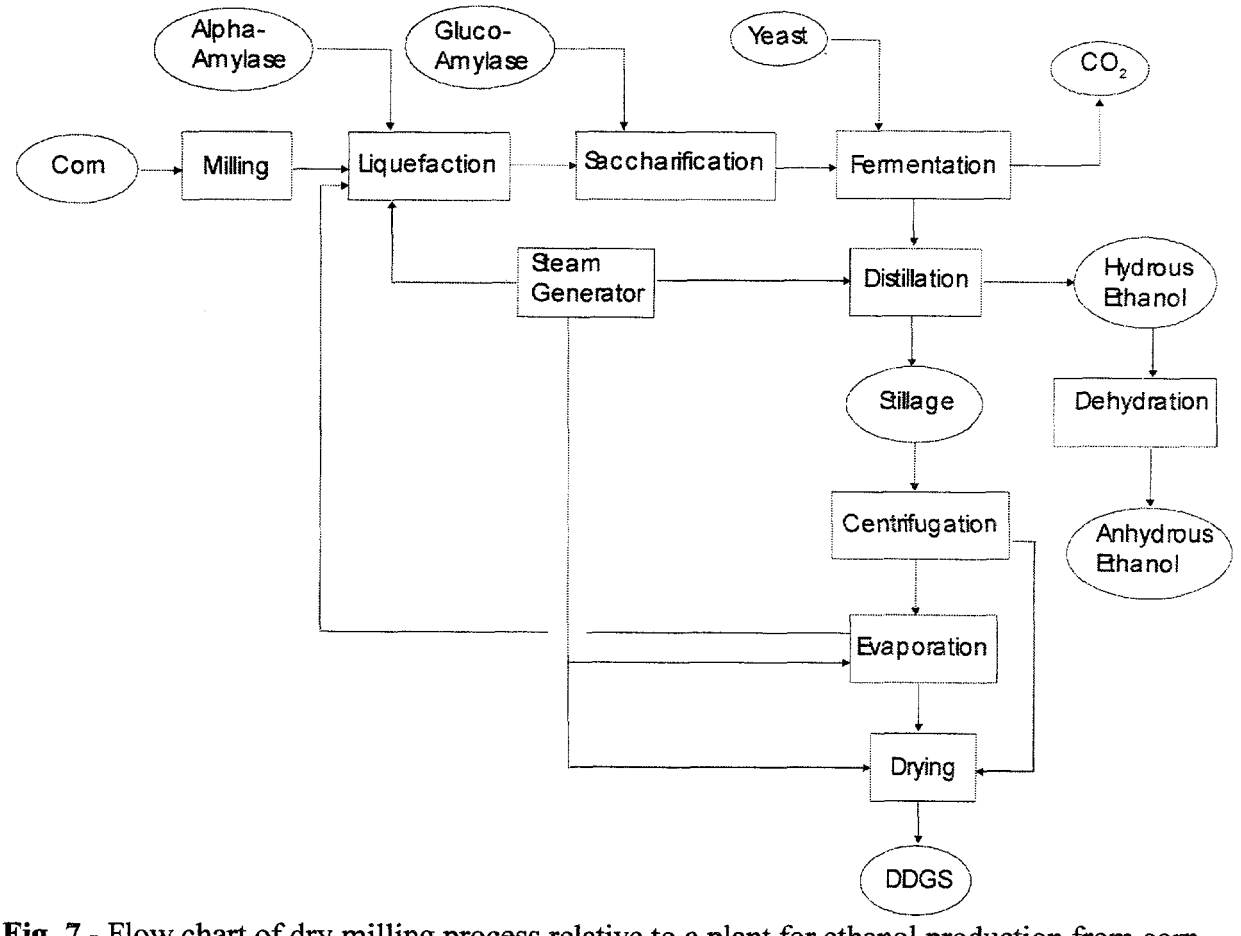


Fig. 7 - Flow chart of dry milling process relative to a plant for ethanol production from corn.

Membrane Technology
Newer than either wet or dry milling, membrane technology was designed to reduce the energy requirements and the processing costs of ethanol production. It is based on developments in the use of membranes to remove and concentrate solvents from dilute solutions. Thanks to progress in this technology, membranes can now be used effectively to process the fermentation liquor left after ethanol distillation, which retains a market value due to the yeast non-fermentable sugars, polysaccharides, acids and proteins it contains (Qureshy *et al.,* 1995).

The membranes can effectively and economically remove some of these high value components for sale as co-products of the fermentation process. Since the solutions can be membrane-filtered without prior concentration by evaporation, the products obtained are not damaged by the high temperatures required for evaporation (Rendleman *et al.,* 1993). In addition, the fermentation liquor can be recycled after filtration, thereby reducing both the volume of water required by the distillation process and the cost of waste disposal (Koseolu *et al.,* 1991).
However, despite the advantage of offering high value co-products, membrane filtration does present some unsolved problems, such as membrane durability and the reduced filtration efficiency that arises when certain substances clog the membranes.

Sequential Extraction Process
This process (SEP) is still not employed on any industrial scale, nor will it be until its technical problems are solved and markets are found for its high protein by-products. By SEP it is possible to extract oil, zain and other proteins in separate stages before the fermentation; it is a two-step process that is self-sufficient in the sense that the ethanol employed is produced by the same plant. In Fig. 8 is reported the flow chart of the process.
Simulations for technical and costing purposes have shown that SEP is an economical way of obtaining 99.5% v/v ethanol from 95% ethanol and could be incorporated into both wet and dry milling systems (Chang *et al.,* 1995; Hojilla *et al.,* 1992). In addition, both the proteins obtained (zein and food protein concentrate) have a high market value. As a hydrophobic protection, the zein can be used in the plastics industry and to make other industrial products, while the food-grade protein could be used in food products.

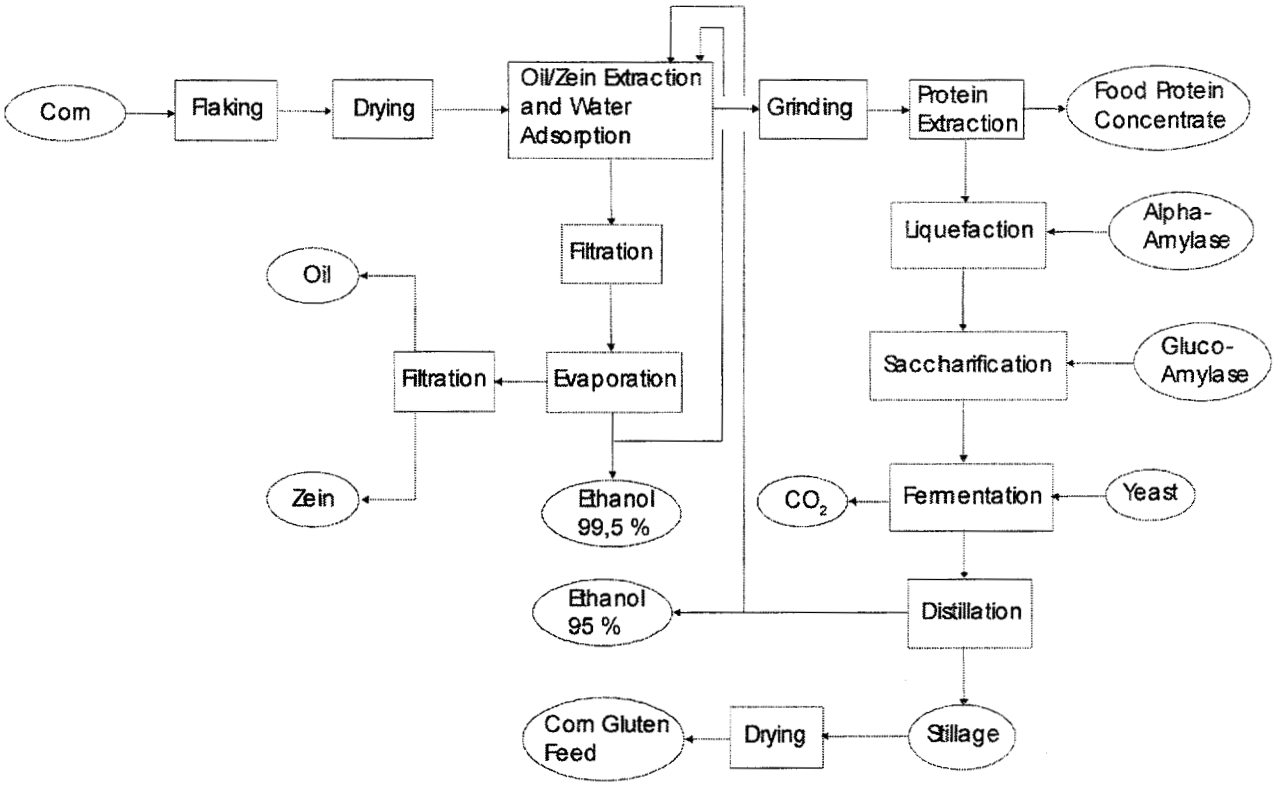


Fig. 8 - Flow diagram for the Sequential Extraction Processing of corn.

ETHANOL MARKET

The word "ethanol" is used so imprecisely that there is no single way to describe the ethanol market. In fact, three different criteria can be used to classify the various product types on sale:

- In terms of feedstock and production process (synthesis or fermentation);
- In terms of composition (anhydrous/hydrous; denatured/non-denatured);
- In terms of use (industrial, fuel, food).

Moreover, the attempts of providing a clear description about ethanol production is in contrast with the difficulty of identifying representative sources for the area.
The Tab. 3 lists data obtained from several sources (journals, agencies).
Although they are officially accredited, some wide differences can be observed in the reported figures: as it is possible to infer from Tab. 3, the differences among the figures describing the same items are remarkable.
Several reasons can be identified to explain these discrepancies.
A first difference lies in the adopted collecting procedure. In fact, beside official sources (national statistical agencies, trans national and international bodies) it also involves industry associations, representative companies, and personal communications. Finally, even educated "guesstimates" or "creative" input are considered if no data are available for a certain Country.
Moreover in many Countries one or two companies control the production of ethyl alcohol. As publicly available figures in sensitive areas could provide foreign rivals with a competitive edge, governments often allow statistical data on trade and production to be suppressed.

Another economic reason for the unreliability of data on alcohol could also be identified: usually, beverage alcohol is heavily taxed giving its production an incentive to smuggle it, or produce it somehow illicitly. That could affect the overall supply picture.
Additionally, there is semantic confusion with regards to the term ethanol. As previously reported, ethanol can be produced from several sources and depending on its production, it can be destined to several uses. Some misleading could be generated by the use of the same word to describe different type of ethanol, to say alcoholic beverages and spirits.

Because of the above reasons, in this report the description on the world ethanol market was given on the base of data only coming from some of the sources mentioned in Tab. 3: Gobi International and F.O.Licht, two of the main comprehensive sources available on the subject.

The biggest producers of ethanol from renewable resources have been those Countries with particularly favourable agricultural and economic conditions. Brazil and the USA were the first to meet both requirements so that they were able to advance from the laboratory or pilot scale to the industrial production of bioethanol (Wheals *et al.*, 1999).
Brazil, in particular, was able to set up programs that also involved the manufacturers of the vehicles the biofuel was essentially intended for, thus stimulating basic research and eventually the production of vehicles designed to run on ethanol alone.

Tab. 3 - Comparison among data on the ethanol production from different sources.

World Ethanol Production

Source	*Year*	*Litres, Bln*
GOBI International [a)]	1998	6.21 [c)]
F.O.Licht (Ch. Berg) [b)]	1998	31.2

Ethanol Production in Europe

Source	*Year*	*Litres, Bln*
GOBI International	1998	3.39 [c)]
F.O.Licht (Ch. Berg)	1998	4.7
CGB (Confederation Generel des Planteurs de Betteraves, France)	1998	2.3

Ethanol Production in Brazil

Source	*Year*	*Litres, Bln*
GOBI International	1998	0.167 [c)]
F.O.Licht (Ch. Berg)	1998	13.5
GOBI International	1999	0.167 [c)]
A.Wheals and L. Basso in *"Fuel Ethanol after 25 Years"* Focus 1999, vol 17, 482.	1999	17.0 [d)]
GOBI International	1996	0.160 [c)]
J. Moreira and J. Goldemberg in *"The Alcohol Program"* Energy Policy 1999, Vol 27, 229.	1996	13.9 [e)]

Ethanol Production in USA

Source	*Year*	*Litres, Bln*
GOBI International	1998	0.56 [c)]
F.O.Licht (Ch. Berg)	1998	6.4
A.Wheals and L. Basso in *"Fuel Ethanol after 25 Years"* Focus 1999, Vol 17, 482.	1998	5.3 [f)]

Ethanol Production in France

Source	*Year*	*Litres, Bln*
GOBI International	1998	0.57
CGB (Confederation Generel des Planteurs de Betteraves, France)	1998	0.70

a) The figures from GOBI Int. includes undenatured ethyl alcohol of an alcoholic strength of 80% v/v or higher, and denatured ethyl alcohol and other spirits of any strength.

b) The figures from F.O.Licht include all categories of ethanol (hydrous; anhydrous; denatured; non-denatured; fuel, industrial and potable).

c) The conversion of tonnes (the original datum reported by the source) into litres was obtained using a density of 0.81 kg/litre, which is the value of hydrous ethanol commercially available at 95% v/v of concentration.

d) This figure is relative to fuel-ethanol production from sugarcane: $10.5 \cdot 10^9$ litres are hydrous ethanol; $6.5 \cdot 10^9$ litres are anhydrous ethanol.

e) Ethanol production from sugarcane.

f) This figure is relevant to anhydrous ethanol production from grains.

In these Countries the production of ethanol from renewable resources was encouraged for the same fundamental reasons:

- To reduce dependence on the oil-producing Countries, something many of the industrialised nations only began to take seriously in the Seventies at the time of the first major oil shock;
- To create new markets to absorb agricultural surpluses or offset fluctuations in demand and provide income for local producers of feedstock (sugar cane in Brazil, grain in the USA and Canada);
- To reduce atmospheric pollution in response to the general public's growing sensitivity to environmental issues.

Most sugar-based (glucose, fructose, saccharose) ethanol derives from sugar cane and sugar beet. In this segment **Brazil** is the undisputed world leader as one of the world's first nations to have promoted biomass-to-ethanol production on a massive scale.
Initially, the production of ethanol from sugar arose as an offshoot of the sugar processing industry. However, the 1974 rise in oil prices created economic and political conditions that led the Brazilian government to promote the industrial production of fuel from renewable resources. The year 1975 saw the launch of ProAlcool, the first government program for that purpose. ProAlcool was actually a two-stage program.
In stage one the plants as already existed in Brazil began to produce hydrous alcohol as an additive for benzine to replace lead tetraethyl. From 1979 on, output was gradually increased so that enough could be produced to meet the needs of the motor vehicles designed to run on hydrous ethanol alone, which had become commercially available in the meantime. Output of these vehicles, in fact, increased dramatically, boosted by a series of factors: several agreements negotiated between government agencies and manufacturers; stable ethanol prices; incentives for sugar cane growers (Moreira and Goldemberg, 1999).

In the meantime, production had been subdivided into anhydrous ethanol (used mostly as an oxygenated anti-knock additive to replace lead tetraethyl), and hydrous ethanol as fuel for the cars specifically designed for it. In 1999 Brazil produced some 10.5 Bln litres of hydrous and about 6.5 Bln litres of anhydrous ethanol.

Most cereal (starch) based ethanol is produced by the USA and Canada. The **United States**, in particular, began to promote ethanol production from renewable sources in 1978, shortly after the launch of the ProAlcool project, spurred on by the encouraging results being obtained in Brazil six years into the campaign. In **Canada** production did not start until 1980.
In both Countries most of the produced ethanol is corn-based, though wheat, oat and barley also made a significant contribution. In the case of the latter cereals, in order to make the ethanol production more economically advantageous, an effort was made to develop co-products with a high market value in order to cover the high cost of both the raw materials and the production process (Tibelius, 1996).

Though bioethanol had been long discussed in **Europe**, concrete results have only recently begun to emerge.

The **EU**'s biggest producer is **France** where some 60% of all biomass-based ethanol derives from sugar beet, which also accounts for around 75% of France's total output of fermentation-based ethanol.
Britain comes second, most of its output being synthesized from fossil hydrocarbons.
In **Germany** too, most ethanol is still synthetic, though it is worth noting that one third of Germany's ethanol is also produced by fermentation, the major feedstocks being potatoes (c. 35%) and cereals (c. 40%) (F.O. Licht).

Italy and **Spain** each contributed some 10% to the total output, though in both Countries quantities varied significantly, since they obtain their ethanol by distilling it from wine. Given its provenance much of this alcohol is used by the food industry and is mostly exported in the form of alcoholic beverages (F.O.Licht).

In **Asia** the main producer Countries are China and India. In **China** about 95% of the output is agro-ethanol, which is mostly used for making alcoholic drinks. In **India** the primary source is molasses. Some 50% of the ethyl alcohol produced is used to make disinfectant spirit for domestic or medical use, while the other half is industrially processed into a wide variety of products. There is currently very little Indian demand for ethanol as fuel. Industrialists are hoping for government programs along the lines of Brazil's "ProAlcool Project", but India's Ministry of Energy seems disinclined to launch any such project on the grounds that India's output of molasses fluctuates too much to guarantee any regular supply of alcohol to the fuel market (F.O. Licht).

South Africa is the major ethanol producer on that continent. Most of its ethanol comes from coal or gas, though a modest amount is fermented from molasses.

ECONOMIC ASPECTS

As soon as the first programs on bioethanol production were launched, the long debate opened on the cost of producing ethanol from renewable resources and the competitiveness of the conversion from fossil fuels. The greatest criticism was reserved for the governmental subsidies and/or tax breaks designed to attract private investors into the biofuel production industry (Vollebergh, 1997; Lunnan, 1997).
The case for biofuels is, at least, debatable: while they are environmentally preferable to fossil fuels, they are so expensive to produce that they represent a very costly alternative to the fossil fuels they are meant to replace. It has been estimated, in fact, that biofuels cost around 3 or 4 times as much to produce as conventional fuels.

The major factor in the high cost of biofuels is the cost of the feedstock required (Wyman and Hinman, 1990). Even if revenues from the sale of co-products offset that cost, biofuels remain more expensive to produce.
In Europe it costs $0.55-0.80/litre to produce ethanol, mostly from wheat and beet, whereas in order to be competitive the product ought to be sold at less than half that cost of producing it.
Things are no better in America where the cost of producing ethanol in a dry milling plant is about $0.5/litre, while the federal subsidy in the 1990s was $0.2/litre and the market price $0.35/litre. These figures seem to suggest that the production of ethanol from biomass is simply not competitive without governmental subsidies (Lunnan, 1997).

In the overall economy of the process several factors help to keep production costs down.
The first of these is on-site re-utilisation of the bagasse. Most Brazilian distilleries employ cogeneration systems to produce the power needed to run their factories (Moreira, 1997). These distilleries incorporate a purpose-built storage facility for the bagasse and the cane waste (mostly leaves and tops) from the treatment the cane is subjected to prior to milling, all of which is burned to produce steam. That steam, in its turn, produces enough electricity, mechanical power and heat to cover the distillery's energy requirements.

If the fermentation step takes not more than 6-10 hours, the ferments can also be recycled and used for a further 200 days or so. Any risk of bacterial contamination can be avoided by the addition of antibiotics to the fermentation broth. In both the continuous and the discontinuous

process, the yeasts are centrifuged out of the fermented *medium* and rinsed with dilute H_2SO_4 to reduce the risk of bacterial contamination before being reutilised for fermentation.

Finally, many Brazilian distilleries dry out some of the re-utilised yeast for sale as cattle feed, the market value of which rises with its protein content. The ratio yeast's protein and carbohydrate can, in fact, be adjusted by an endogenous fermentation process, at the end of which the dry yeast may have a protein content of around 40% w/w (Wheals, 1999).

In the USA, the world's biggest producer of ethanol from corn, wet and dry millings are the most widely employed technologies. For economic reasons, some 60% of U.S. ethanol distilleries employ the wet milling system, since the revenue from the sale of its co-products is sufficient to cover 50% of the cost of the corn they process.
America's corn-based fuel ethanol industry is heavily reliant on governmental subsidies and tax credits. In fact, it is generally agreed that the fuel ethanol industry will not survive unless it is able to develop by-products with a higher market value than those currently produced which are generally sold for animal feed.
The most important and most variable cost factor in fuel ethanol production is its net cost,[1] hence the search for high value markets to absorb the industry's by-products.
That problem becomes all the more urgent when the ethanol is produced from cereals like wheat, barley or oat which are inherently more expensive given their higher nutritional value as human food products. That cost factor, moreover, is exacerbated by the fact that given these cereals' lower starch content, their ethanol yield is also indeed lower.

Moreover, maize is not the most important cereal crop in many Countries (Europe, Australia, Canada); for them the production of ethanol from cereals will only be economically viable if are developed technologies that allow for the incorporation into the ethanol production the extraction of co-products with a higher commercial value than animal feed. Ideally, that means products for use in the human diet, as well as cosmetics and pharmaceutical products.
A planned approach to these aspects could help to promote the bioethanol industry.

[1] The net cost of corn is the difference between its price to the distillery and the profit from the sale of distillery by-products.

BIOETHANOL
AS ALTERNATIVE TO CONVENTIONAL LIQUID FUELS

Ethyl alcohol is a colourless fluid completely soluble in water. Highly inflammable, it has been used as motor vehicle fuel for decades. Indeed, the Energy Policy Act (USA, 1992) recognises and defines it as an alternative fuel and in several Countries it has become a potential rival to conventional fuels, being used to power ships, buses and heavy duty engines. Though ethanol is more costly to produce than petrol, the ever-increasing price of oil and the need to make more use of energy resources that meet our planet's own need for sustainable development are focusing attention on the search for competitively costed ways of producing biofuels. Most ethanol is produced by the natural fermentation of sugars derived, directly or indirectly, from vegetable biomass (hydrolysed starch, cellulose and hemicellulose), only 7% being synthesized from coal or oil (Berg, 1999). Since ethanol is produced almost entirely from renewable resources, it looks like a viable alternative to conventional fuels: in fact, any CO_2 emissions generated by its use would be reabsorbed by the crops planted for its production, thereby helping us to achieve the CO_2 emission targets established by the international agreements, such the Kyoto protocols (Fig. 9). Tab. 4 and 5 list some significant chemical and physical properties of ethanol and compares them with those of other fuels.

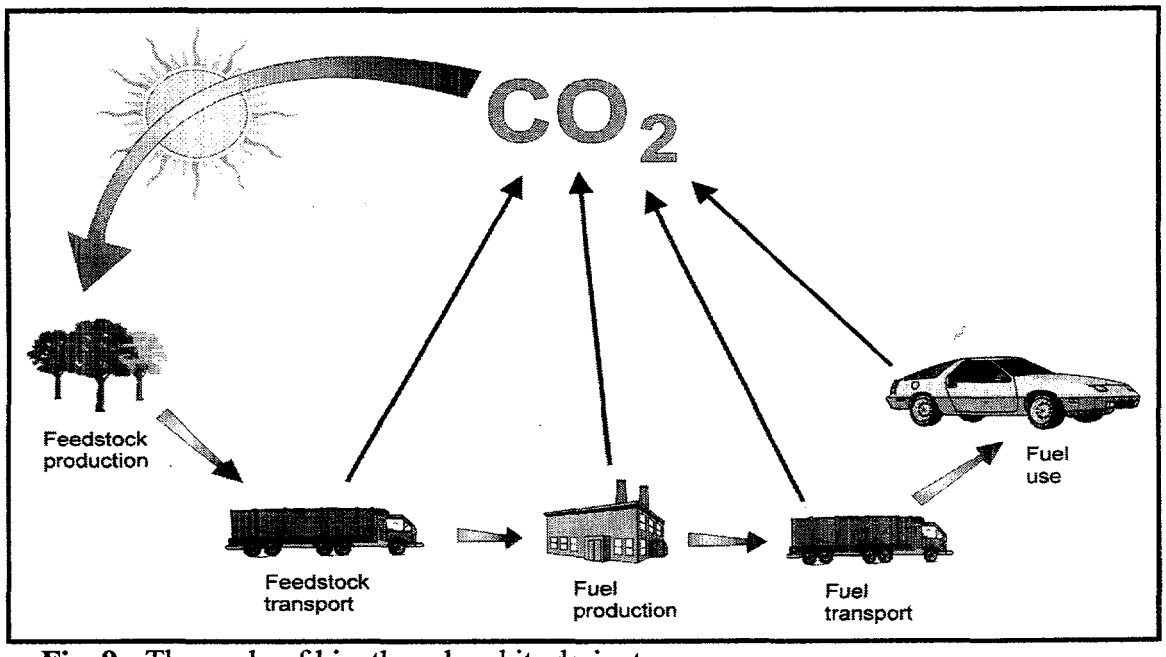


Fig. 9 - The cycle of bioethanol and its derivates.

Tab. 4 - Chemical and physical properties of relevant fuels.

	Gasoline	MTBE	Ethanol	Benzene	Toluene
Molecular weight (g/mole)	~100	88.15	46	78.11	92.13
Specific gravity	0.72-0.74	0.74	0.79	0.88	0.87
Boiling temperature °C	22.5-27	53.6-55.2	78.5	80.1	110.6
Water solubility (mg/L)	100-200	43,000-54,300	Infinite	1,780	534.8
Vapor pressure at 25°C (mm Hg)		245-251	49-56.5[b]	95.19	28.4

Tab. 5 - Physical properties of ethanol, petrol, and diesel oil.

	Ethanol	**Petrol**	**Diesel oil**
Energy density[a] (Mj/Kg)	26.6	43.8	42.8
Energy density[a] (Mj/l)	21.0	32.0	36.4
Flame temperature[b] °C	1930	1977	2054
Octane number[b]	96-113	85-96	n.a.
Specific Energy[b]	3.00	2.92	-

a) Source: The World Bank (1980); b) SAE Technical Paper No. 930376 © 1993 Society of Automotive Engineers, Inc.

The difference in energy density has little impact on engine performance, but parameters like fuel weight and fuel tank capacity do affect fuel economy (*i.e.* the available ratio Km/l).

In the case of using ethanol as combustion fuel, some estimates suggest that the extra weight caused by the lower energy density of ethanol makes ethanol-powered engines about 1% less efficient (Bailey, 1993). However, its lower flame temperature reduces engine heat loss and enhances thermal efficiency. Ethanol's dimmer flame also further reduces heat loss through radiation and cuts the production of NO_x by the engine (Owen, 1990). Thanks to its high H/C ratio, ethanol produces more gas during combustion than either petrol or diesel oil, which means higher pressure and hence greater piston effort (about 7% more than petrol and 1% more than diesel). The specific energy figure is an indicator of the heat released by the various fuels: the higher the figure, the greater the engine's power output. Meanwhile, octane number is related to compression ratio: the higher the number, the greater the efficiency and the power of the engine and here ethanol is 6-10% better than petrol.

Ethyl alcohol can be used as biofuel either in its pure state (E100) or blended with petrol in various proportions, so E85, E95, and E10 indicate that the blend is 85%, 95% or 10% ethanol. Low concentrations of ethanol are generally used for antiknock or oxygenation purposes: when used in higher proportions or in its pure state (E100), this type of biofuel is more or less competitive *vis à vis* its conventional alternatives depending on the extent to which the engines it powers have been optimised to benefit from its properties. Valve timing and compression ratios have to be modified and several components susceptible to corrosion, like fuel tanks and connectors, have to be replaced. Ethanol can also be used as a raw material for the production of ETBE (ethyl tertiary butyl ether), which is a compound obtained from ethanol and isobutylene used as a petrol additive. ETBE is an "octane enhancer", *i.e.* it increases the fuel's octane number and oxygenates it so that it burns more completely and therefore has a lower environmental impact. In fact it offers a whole series of environmental benefits:

- *It reduces ozone levels in the lower layers of the atmosphere*

The ozone causes respiratory problems and damages many plants. However, it does not filter upwards into the stratosphere where ozone serves the useful purpose of protecting us from the sun's ultraviolet rays. There are many compounds that react with sunlight to form the ground level ozone that combines with dust and other particles in the air we breathe to form "smog". These compounds include carbon monoxide, incombusted hydrocarbons, benzene and nitrogen oxides. The use of oxygenated fuels like ethanol reduces ozone formation because the emissions produced by its combustion are less reactive with sunlight than the emissions from petrol combustion.

- *It reduces the greenhouse effect*

Certain gases increase the greenhouse effect on the earth's atmosphere to produce a constant rise in the earth's temperature. These gases are essentially carbon dioxide, methane and nitrogen oxide. In normal conditions, the use of ethanol-petrol blends like E85 (85% ethanol, 15% petrol) or E10 (10% ethanol, 90% petrol) can reduce gas emissions by 37.1% and 3.9% respectively.

- *It reduces the emissions from ethanol blends*

Thanks to the way ethanol promotes almost complete combustion, adding 10% ethanol to other fuels reduces their carbon monoxide emissions by 25-30% and their carbon dioxide by 6-10%. In fact the production and use of ethanol releases less carbon dioxide than is absorbed by the plants used to produce it. In the case of the volatile organic compounds (VOCs), that are a primary source of ground level ozone, the fact that ethanol oxygenates the fuel means that even a very low ethanol content will reduce VOC emissions by 7%, a figure that can rise to 30% in blends with a high ethanol content. Finally, since ethanol contains no sulphur and promotes total fuel combustion, its use also cuts sulphur dioxide emissions.

- *Benefits from the crops grown for ethanol production*

Ethanol is produced from biologically renewable resources like starchy or sugar-rich crops. In energy terms, the ethanol contains about 23.6 (high heating value) Mj per litre. However, given its high combustion efficiency and its octane credit, it is the equivalent of 28 Mj petrol. Add in the 3.9 Mj/litre of energy supplied by ethanol co-products, and we get 31.9 Mj/l. It takes 4.9 Mj (15% of the ethanol's energy) to grow and 14 Mj (44% of the ethanol's energy output) to convert the corn needed to produce one litre of ethanol using current technologies. Hence the energy content of the ethanol produced is almost double the amount used up in growing and converting the raw material and that figure is bound to rise as technologies are improved.
Bull *et al.*, (1994) studied the atmospheric emissions of E95 (95% ethanol, 5% petrol) and compared their findings with emissions from reformulated gasoline. Tab. 6 shows the change in mass emissions of E95 compared to reformulated gasoline.

Tab. 6 – Percent change in mass emissions of E95 fuel compared to reformulated gasoline (Bull *et al.*, 1994).

	-90	-80	-70	-60	-50	-40	-30	-20	-10	0	10	20	
CO										■			Boiler burns plant wastes in ethanol production
SO_2			■										Ethanol has no sulfur
NO_x										■			
VOC								■					Evaporative losses are lower
CO_2	■												Carbon is recycled in regrowth of the plants

BIOETHANOL FROM LIGNOCELLULOSIC BIOMASS

The nature of the biomass raw material is a major factor when it comes to working out the cost and the equipment required for any hypothetical production of ethanol. The simplest way is to start with biomass that already contains a satisfactory quantity of the monomeric sugars that can be directly fermented into ethanol, as it is the case of sugar cane and sugar beet More often than not, however, the sugar in the biomass is in a biopolymeric form that requires pre-treatment to transform the sugar content into an accessible monomeric form. Glucose polymers include starch and cellulose, while hemicellulose is a polymer that is largely composed of sugars containing 5 carbon atoms, such as xylose. **Starch** is currently a major source of ethanol. As

previously reported, it is contained in cereal grains (*e.g.* corn) and it is composed of glucose chains linked together by □-1,4 and □-1,6 glucoside bonds. The random sequence of these two different bonds makes the polymer amorphous, *i.e.* non-crystalline in structure, so that *it is* easily accessible to the enzymes that can break it down into its constituent monomers. **Cellulose** is the major component of lignocellulosic biomass and consists of glucose linked together to form chains by □-1,4 bonds. The entire polymer structure is ordered, rigid and compact, so that in order to obtain free glucose, the biomass has to undergo pre-treatment that deconstructs its fibre to facilitate the action of the enzymes.

Hemicellulose is a short, branched polymer composed partly of sugars with 5 carbon atoms, such as xylose and arabinose and partly of 6-carbon sugars like glucose, mannose and galactose. Being branched, this polymer is also amorphous and easily hydrolysed. Hardwood hemicellulose contains more xylose while 6-carbon sugars prevail in the softwood type (Kuhad, *et al.,* 1997). Since most starchy and sugary biomasses are used as food, attention has focused on the exploitation of the cellulose and hemicellulose sugars in lignocellulosic biomasses to produce ethanol. Trees and waste from farming and forestry are a practically limitless and renewable source of cellulose and hemicellulose. They also contain **lignin,** a three dimensional polymer with a structure consisting of phenylpropane units held together by ether and carbon-carbon bonds. The lignin is structurally interwoven with the plant's cellulose and hemicellulose polymers and provides the rigidity of the plant. Lignocellulose biomass also contain smaller amount of other compounds generally known as **extractives** (terpenes, fats and waxes, phenolic compounds, lignans) and inorganic salts that are determined as **ash**

Fig. 10 shows the chemical structures of cellulose, hemicellulose and lignin; Fig. 11 shows the main constituents of the extractives.

Tab. 7 shows the composition of some biomass types together with a stoichiometric calculation of the quantity of ethanol theoretically obtainable from 1 kg dry biomass of each type.

Cellulose generally accounts for 30-60% of the biomass weight (higher in woods, lower in agricultural waste). The Hemicellulose content varies from 10% to 40%, the lignin content from 10% to 25%

The use of biomass as a source of energy was forcefully supported at the Kyoto Conference on the grounds that the CO_2 produced by the combustion or processing of these materials is absorbed by the biomass sources via their photosynthesis. A further advantage of biomass is that it is widely and easily available; indeed when, as it is often the case, the biomass grows no more than 50 km away from its processing plant, the energy consumed by its transportation is no more than 3-5% of its energy content (Schlamadinger, *et al.,* 1997). The amount of CO_2 emitted will depend on the type of transportation involved: road, rail or water. A marine engine in fact emits 4 times as much CO_2 as a truck's and 3 times as much as a train's (Avella and Gerardi, 2000).

In the 15 EU Countries biomass covers 3.5% of energy demand and 90% of it comes from forest waste (Chartier, 1996). Forestry supplies 55% of the renewable resources for energy uses. It is expected that the contribution of biomass will become increasingly important in the near future, rising to 6% of all renewable resources in the early years of this century and doubling to 12% by 2010 (White paper on energy sources EEC, 1997). Europe's biomass is mostly used for heat production at present with about 1,500,000 Gj produced annually.

The white paper predicts that over the next 15 years, at least 10 million ha of land will become available for energy crops and would be able to produce about 1,000,000 Gj/y. More land would become available if Eastern European Countries are admitted to the EU and in response to rising demand for food crops in the Countries of the Southern Mediterranean area. Much of this land could be assigned to ethanol-producing crops. However, it should be considered that the production of biofuels from biomass is less efficient than the use of the same biomass to produce heat, at least in energy balance terms.

Forest waste could produce some 2,000,000 Gj/y and the main obstacle to its use is the fact that in most cases biomass is more expensive to use than fossil fuels. However, environmental requirements or the local availability of wood-chip in certain areas may reverse this situation (The European Commission, 1998). A number of proposals have also been advanced that could improve the economic viability of biomass as a source of energy. One idea is the conversion of deciduous woodland into "tall tree" plantations, which would involve cutting down the deciduous trees, using their low value timber to produce energy and replanting the area with selected "tall trees" (Avella *et al.*, 1998). This would offer the added benefit of preventing the fires that deciduous woodland is particularly susceptible given its inherently shrubby character. The European Countries most interested in this possibility are those in the Mediterranean area, especially France that has vast expanses of neglected deciduous woodland.

Tab. 7 – Typical composition of several lignocellulosic biomasses and their potential ethanol outputs.

	Ash	**Hemicellulose**	**Cellulose**	**Lignin**	***Ethanol potential***
	%	**%**	**%**	**%**	***kg/kg****
poplar		17	49	18	*0.37*
eucalyptus		31.8	43.3	24.7	*0.42*
maize stalk straw	3	26	38	11	*0.36*
wheat straw	1.3	27.6	34	18	*0.35*
rice straw	18.9	22.7	37	13.6	*0.34*
oat straw	2.6	24.9	37.1	15.4	*0.35*
rye straw	1.2	25.7	37.1	17.6	*0.35*
Barley straw	7.1	44	37	11	*0.46*
potato rests	5	11.8	26		*0.21*
miscanthus straw	2.7	29.6	44.7	21	*0.42*
kenaf			41.9	12.3	*0.24*
hemp (wood fiber)		27.5	37.5	22	*0.37*
beet tail and beet green	5	10	10	5	*0.11*
tobacco stalk	2.4	28.2	42.4	27	*0.40*
wood, ailanthus	0.5	26.6	46.7	26.2	*0.41*
Soybean stalks and leaves		18.5	32.1		*0.29*
Bagasse		24.6	39.7	25.2	*0.36*

(Source: Phyllis database for biomass and waste)

** calculated from the processes: 1) cellulose □ glucose □ ethanol; 2) hemicellulose≅xylane□ xylose □ ethanol*

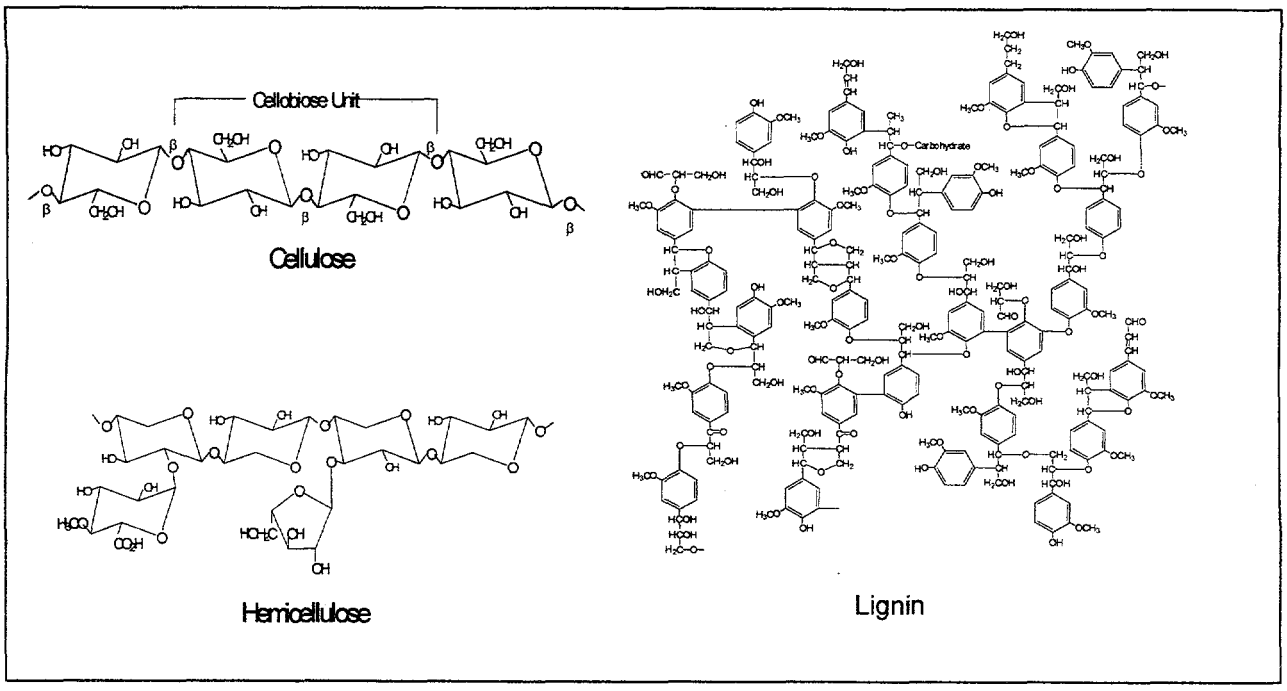


Fig. 10 - Chemical structure of cellulose, hemicellulose and lignin (Fan *et al,* 1987).

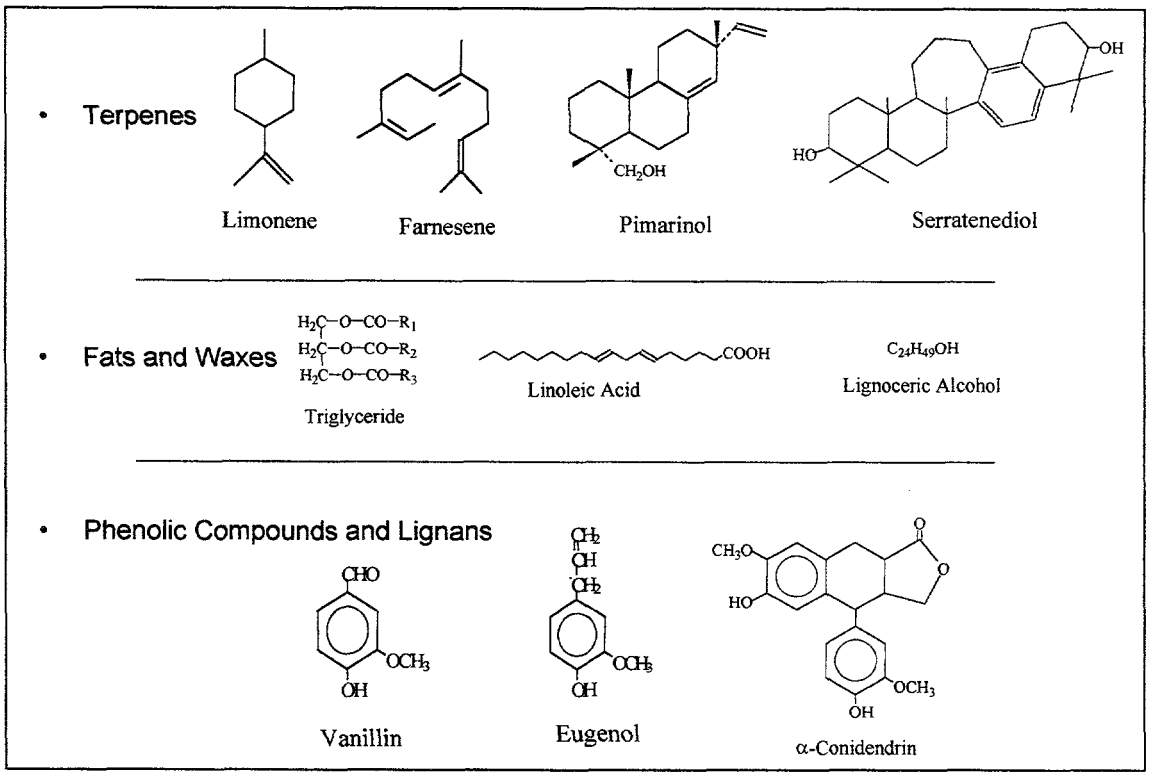


Fig. 11 – Chemical structure of the extractives' components

BIOMASS AVAILABILITY IN EUROPE

The word biomass is used to describe all material deriving from the organic photosynthesis of CO_2. That biomass consists of the products and waste from agriculture and forestry as well as the products of their processing into foodstuffs and industrial products (lignocellulose, starch, sugar, oil). Biomass also includes the organic fraction of municipal solid waste.
Since plants grow all over the planet, biomass represents the most abundant renewable organic matter available to us for energy or industrial production. It has been estimated that the Earth's land masses produce 120 Gt of this material every year of which 70% comes from forests, 20% from savannah and prairie land and 10% from crops. Meanwhile, the Earth's waters account for half as much again, of which 90% is from plankton and microalgae and just 10% from macroalgae (Avella and Gerardi, 2000).

Turning to cereal crops, the EU's biggest producers of wheat and barley are France and Germany, whose 1999 output came to about 37,000 and 19,000 Gt respectively. All EU Countries grow both these crops, as well as oat. A good deal of maize is also grown in some Countries (15,000 Gt in France, 10,000 Gt in Italy). But it is not a major crop in all Countries. Tab. 8 gives details of the cereals grown in the 15 EU Countries in 1999, including outputs and areas under cultivation.

Tab. 9 shows outputs of other herbaceous crops in the EU. The most abundant include sugar beet and potatoes (mostly in Germany and Holland) and tomatoes with Italy accounting for nearly 50% of the total crop. Tobacco and artichokes are only minor items.

Turning to fruit trees, almost all EU Countries boast vast apple and pear orchards, as well as vineyards, with France, Germany and Spain among the biggest producers. Citrus fruit and olives are major crops in Spain, Italy and Greece. Tab. 10 provides detailed figures.

Apart from the main crop products that go for food, there is also a vast quantity of by-products that are often treated as waste. These may be the stems and leaves of herbaceous plants or the pruning of fruit trees. In addition, since the usable part of cereal crops consists of grains, there remains an abundant quantity of straw as their by-product or waste. It is precisely this waste (residual biomass) that can become additional raw material for the production of liquid fuels like ethanol.
In order to get some idea of the availability of agricultural by-products, a by-product/main-product coefficient has been calculated (Tab. 11). The calculations were based on official statistics from ISTAT; EUROSTAT, FAO for a 4 years period in order to minimise the effects of annual fluctuations (De Castro *et al.,* 1991).

The Tab. 11 shows the quantity of waste/by-product available in EU Countries, obtained by multiplying the main products/by-products ratio by the output (in Gt) of the various crops. For example, the entire EU produced 97,766 Gt of wheat in 1999, which also produced about 68,443 Gt of straw. If it is assumed that the total mean percentage cellulose/hemicellulose content of straws comes to around 62% of the total crop, straws alone could provide with a potential raw material amounting to around 42,435 Gt from straw alone (Tab. 7). If all this straw were converted into ethanol, the theoretical yield would be around 23,955 Gt ethanol, this figure would, of course, be multiplied if, instead of wheat-straw alone, we examine all agricultural waste.

Tab. 8 - Cereals, outputs and areas under cultivation in EU Countries in 1999. *(Source: FAO)*

	wheat		barley		oat		rye		paddy rice		maize	
	Mt	Area Harv (Ha)	Mt	Area Harv (Ha)	Mt	Area Harv (Ha)	Mt	Area Harv (Ha)	Mt	Area Harv (Ha)		Area Harv (Ha)
Austria	1,285,000	260,579	1,215,000	243,886	150,000	35,503	223,000	55,901			1,728,000	177,189
Belg.-Luxem.	1,634,000	210,000	356,000	65,000	35,000	10,000	8,000	2,500			245,000	26,000
Denmark	4,444,000	631,000	3,619,000	715,000	137,000	27,000	237,000	51,000				
Finland	323,000	128,000	1,788,000	596,000	1,215,000	388,000	32,000	10,000				
France	37,009,248	5,115,195	9,548,000	1,534,000	550,000	122,000	184,000	40,400	100,930	16,970	15,628,000	1,762,600
Germany	19,684,000	2,609,444	13,322,000	2,212,880	1,347,394	266,974	4,319,000	746,982			3,035,800	363,242
Greece	1,900,000	837,900	414,000	128,600	92,000	44,000	35,000	15,000	210,000	22,400	1,900,000	209,800
Ireland	611,000	75,000	1,275,000	190,000	136,000	20,000	400	200				
Italy	7,742,708	2,382,566	1,329,107	349,029	346,013	143,296	12,161	3,974	1,362,452	220,795	9,996,219	1,030,603
Netherlands	1,000,000	137,000	240,000	38,000	10,000	2,000	25,000	5,000			58,000	7,000
Portugal	400,000	242,000	43,235	32,776	74,656	72,317	51,981	50,756	158,539	25,399	1,092,000	190,000
Spain	5,083,800	2,422,400	7,398,900	3,119,000	530,800	409,500	219,700	122,200	845,100	112,100	3,777,000	399,200
Sweden	1,779,400	295,070	1,908,800	495,710	1,093,800	316,490	115,000	24,020				
Un.Kingdom	14,870,000	1,847,000	6,510,000	1,178,000	540,000	92,000	44,000	7,600				
TOTAL UE	**97,766,156**	**17,193,154**	**48,967,042**	**10,897,881**	**6,257,663**	**1,949,080**	**5,506,242**	**1,135,533**	**2,677,021**	**397,664**	**37,460,019**	**4,165,634**

Tab. 9 - Output and cultivated area of herbaceous crops in EU Countries in 1999. *(Source: FAO)*

	sugar beets		potato		tobacco		sunflower seed		Beans green		peas green		soybean		tomato		artichoke		cauliflowers	
	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha	Mt	Are Ha	Mt	Area Ha	Mt	Area Ha
Austria	3,000,000	47,047	660,000	23,180	220	108	58,000	24,249	9,500	500	10,000	1,100	44,500	18,541	18,500	180				
Belg-Lux.	6,150,000	104,000	2,700,000	55,000	1,275	375			60,000	5,000	165,000	8,700			300,000	900			90,000	5,300
Denmark	3,567,000	63,000	1,476,959	38,002							80,000	8,000			18,800	100			6,700	700
Finland	818,000	34,000	756,000	40,000					21	9	5,206	2,036			31,455	119			4,051	440
France	32,776,000	443,824	6,475,000	170,000	26,348	9,040	1,871,000	799,000	127,300	11,359	577,200	34,778	272,000	102,155	904,882	9,445	73,269	13,140	453,616	34,272
Germany	27,586,800	487,583	11,419,908	297,684	8,504	3,522	81,320	32,465	44,328	4,150	23,772	3,990	1,030	396	40,000	329			157,146	5,820
Greece	2,350,000	42,100	900,000	47,500	126,000	67,200	40,413	34,600	70,000	8,500	9,000	1,500	4,000	2,000	2,060,000	47,400	23,000	2,300	65,000	3,400
Ireland	1,400,000	33,000	500,000	18,500					2,200	200	6,200	1,000			7,000	100			8,000	1,100
Italy	14,100,000	282,647	2,076,743	86,858	132,509	47,475	427,872	197,962	198,119	22,221	135,000	22,000	900,905	253,063	7,090,806	130,277	472,228	50,955	448,044	19,671
Netherlands	5,504,500	114,000	8,200,000	183,000					45,000	5,200	60,000	4,600			495,000	1,300			34,000	3,200
Portugal	419,540	8,500	1,150,000	82,000	6,500	2,600	30,143	53,735	38,000	4,000	5,000	1,100			1,175,633	17,281			30,000	1,500
Spain	7,997,700	137,700	3,311,900	134,900	45,000	17,000	555,500	873,700	274,200	21,200	54,100	9,000	9,200	4,200	3,840,400	64,100	254,300	18,100	367,900	19,300
Sweden	2,571,000	58,700	986,000	34,000							47900	9000			20,000	61			8,000	365
Un. Kingd.	10,328,000	185,000	7,100,000	177,600					22,000	1,800	380,200	38,500			117,300	300			188,900	13,600
TOTAL UE	**118,568,540**	**2,041,101**	**47,712,510**	**1,388,224**	**346,356**	**147,320**	**3,064,248**	**2,015,711**	**890,668**	**84,139**	**1558578**	**145,304**	**1231635**	**380,355**	**16119776**	**271,892**	**822,797**	**84,495**	**1,861,357**	**108,668**

Tab. 10 - Output and cultivated area of fruit trees in EU Countries in 1999. *(Source: FAO)*

	grapes		**olives**		**apples**		**pears**		**Peaches and Nectarines**		**Citrus**		**almond**		**filbert**	
	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha	Mt	Area Ha
Austria					395300	11,259	132394	3,000	8036	1,200						
Belg-Luxemb.	351,412	48,000			562,382	9,600	163,200	5,100								
Denmark	22,000	1300			67,000	1600	5,800	425								
Finland					10500	452										
France	8,000,913	870,914	15,989	13,281	2,643,131	78,000	299,880	14,000	477,510	23,745	31,851	2,974	4,500	1,700	4,604	2,424
Germany	1,648,000	101,623			2,062,000	81,000	357,700	25,600	18,000	6,410						
Greece	1,200,000	124,000	1,900,000	757,600	360,000	13,700	75,000	9,765	500,000	52,500	1,199,200	60,020	35,000	39,900	2,500	1,800
Ireland					10000	500										
Italy	9,731,580	870,502	3,207,990	1,101,557	2,416,175	65,052	854,047	47,681	1,805,369	95,215	3,122,744	175,862	114,650	88,938	118,626	68,740
Netherlands	200	30			518,000	14,700	141,000	6,000								
Portugal	550,000	252,000	262,000	286,000	234,684	23,954	135,905	13,314	92,240	10,715	263,478	26,245	11,252	40,826	681	956
Spain	4,969,300	1,163,000	3,202,500	2,200,000	922,200	50,000	730,600	41,000	986,800	70,000	5,609,200	283,350	262,900	664,000	27,900	28,000
Sweden					65,000	6,550	15,500	1,200								
United Kingdom	1400	840			249900	12,800	22700	2,300								
TOTAL UE	**26474805**	**3,432,209**	**8588479**	**4,358,438**	**10516272**	**369,167**	**2933726**	**169,385**	**3887955**	**259,785**	**10226473**	**548,451**	**428302**	**835,364**	**154311**	**101,920**

Tab. 11 – Availability of biomass by-products in EU (De Castro, P. *et al.*, 1991)

	Main product	By-product or waste	Ratio by-product/ main-product	Potential by-product in EU (Gt/y)
common wheat	grain	straw	0.69	
durum wheat	grain	straw	0.7	68,443
barley	grain	straw	0.8	39,180
oat	grain	straw	0.7	4,380
rye	grain	straw	0.7	3,854
rice	grain	stalks	0.67	1,794
grain maize	grain	stalks	1.3	48,698
grain maize	grain	cobs	0.2	7,492
sugar beet	roots	leaves and collars	0.35	41,499
potato	tubers	stems and leaves	0.4	19,085
tobacco	leaves	stems	1	346
sunflower	achenes	stalks	2	6,128
beans and peas	grain	stems and leaves	1.5	3,674
soybean	grain	stems and leaves	1.5	1,847
tomato	berries	stems and leaves	0.3	4,836
artichoke	capitula	stems and leaves	2.5	2,057
legume fodder crops	seeds	stems and leaves	2.5	-
cauliflowers	Inflorescences (or capitula)	stems and leaves	2.5	4,653
grapevine	berries	tendrils	0.41	10,855
olive tree	stone fruit	wood and branches	1.15	9,877
apple tree	fruit	waste wood	0.1	1,052
pear tree	fruit	waste wood	0.1	293
peach tree	stone fruit	waste wood	0.2	778
citrus	hesperidia	waste wood	0.1	1,023
almond tree	seeds	waste wood	1.9	814
filbert tree	filberts	waste wood	1.9	293

Energy crops in Europe

In Europe, the biomass used to produce heat and electricity is mostly forest, farm and agroindustrial waste. Biofuels, on the other hand, whether for use on their own or for blending with conventional fuels, are almost exclusively made from starchy or sugary crops like cereals and sugar beet or from oil seeds crops like colza or sunflowers. However, the increasing need to make use of renewable resources in producing energy is focusing attention on a search for targeted crops whose products or by-products would be used exclusively for the production of biofuels. A number of organisation and research units in the various EU Countries are therefore working on the development of "energy crops" whose biomass could be exploited on a vast scale. However, while the science of producing, processing and using these crops is well established, widely known and undisputed, it has only been put into practice on a very limited scale and in specific situations that are not easy to compare in technical or economic terms, quite apart from the production cost problems (Pignatelli, 1997).

The Tab. 12 presents data on land devoted to energy crops in Europe, whether on an industrial scale or for demonstration purposes only.

Tab. 12 - Area devoted to energy crops in Europe.

	Area, Ha
Colza	800,000
Eucalyptus	500,000
Sunflower	55,000
Salix	18,000
Triticale and other cereals	9,400
Sugar-beet	6,250
Phalaris	4,050
Populus	550
Hemp	350
Miscanthus	170
Kenaf	65
Cardoon	55

(Source: summary of report on European Energy Crops Overview Project, EC-FAIR concerted action FAIR1-CT95-0512, September 1996)

Naturally, these energy crops are not uniformly distributed all over Europe; moreover given the differences in terrain, climate, agro-industrial system of the various regions, not to mention legislative differences that reflect varying levels of interest in the use of these raw materials as a source of energy, it is inevitable that some crops are considered important in the Mediterranean Countries, but of no interest at all in Central and Northern Europe and *vice versa*. There are however crops that adapt well to very different environments and are widely cultivated. Tab. 13 lists the main crops considered to be of actual or potential interest for biomass or biofuel production in the European Countries (Pignatelli, V., 1997).

As the Tab. 13 shows, the few examples of industrial use are: the colza and sunflower crops used for biodiesel in Austria, Germany, France and Italy; the ethanol produced from common wheat and sugar beet in France; the production of heat and electricity from the willow and phalaris biomass grown in Sweden, or eucalyptus in Portugal.

Tab. 13 - Main (actual or potential) energy crops grown in EU Countries (Pignatelli, 1997).

	COUNTRY													
Colture for biomass	DK	S	SF	A	D	IR	UK	B	NL	F	ES	GR	I	P
Broom													▒	
Cardoon											▒	▒	▒	
Common reed													▒	
Eucalyptus										▒		▒	▒	■
Hemp				▒					▒					
Kenaf													▒	▒
Miscanthus	▒			▒	▒	▒	▒	▒	▒			▒	▒	▒
Phalaris		■	▒											
Poplar				▒	▒	▒	▒	▒	▒	▒	▒		▒	
Robinia													▒	
Salix	▒	■	▒	▒	▒	▒	▒	▒	▒				▒	
Sorghum for fiber										▒			▒	
Triticale and other cereals	▒			▒										
crops for biofuel														
Colza	▒		▒	■	■	▒	▒	▒		■			■	
Common wheat					▒					■				
Sorghum					▒			▒			▒	▒	▒	▒
Sugar-beet					▒	▒				■			▒	
Sunflower				■									■	
Tomato					▒									
Jerusalem Artichoke											▒		▒	
Triticale					▒									

▒ Crops of potential interest
■ Crops in industrial use

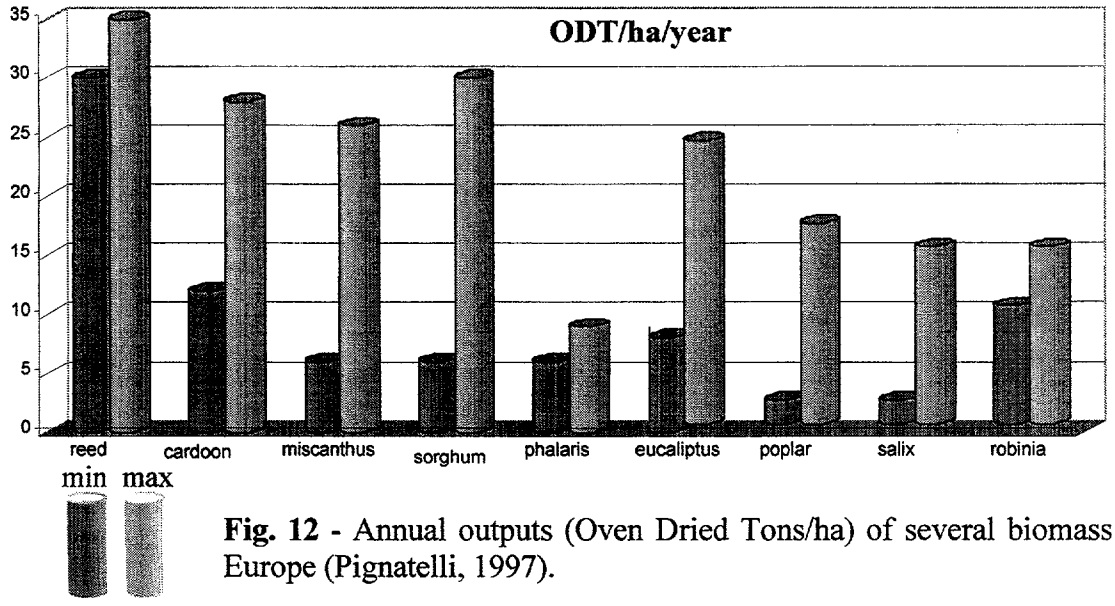


Fig. 12 - Annual outputs (Oven Dried Tons/ha) of several biomass crops in Europe (Pignatelli, 1997).

Sunflower *(Helianthus annuus)*
A popular ornamental plant, the sunflower is a native of the temperate regions of North America where it was originally grown for its edible seeds. Later, the seeds were used as a source of oil in Russia. Subsequent breeding for crop purposes has concentrated on shortening the stem, discouraging branching, and latterly on the development of high-yielding hybrids. Today the sunflower is a worldwide source of edible vegetable oil, the biggest producers being Russia, Argentina, Eastern and Western Europe, China and the USA. In some parts of Europe, especially France, sunflower oil is put to industrial use, including the production of biodiesel. Over 2,000,000 hectares are currently devoted to this crop in the 15 EU Countries, which produce over 3,000 Gt of seed every year.

Jerusalem artichoke *(Helianthus tuberosus)*
Small areas of the USA and Europe have been growing these plants for their tubers in recent years, but the market has remained very small. The tubers do contain a high concentration of inulin (a fructose polymer) from which syrups can be obtained for use by the food industry and for ethanol production. In addition the large quantities of biomass provided by the stems can be used as animal fodder or as an energy resource.

Oilseed rape *(Brassica napus ssp. Olifera)*
This European native has only recently been grown on an extensive scale. Planted as a pioneer crop in reclaimed marshes or wasteland in the past, its seeds are used to supply oil for lamps and lubrication. Recently, growers have concentrated on varieties with a minimum erucic acid content in the seeds, for use as cooking oil or in the production of margarine. On the basis of research conducted in Austria, this oil is now also being used as a less polluting alternative to diesel oil (biodiesel). In 1999 the EU Countries produced over 11,000 Gt rape on a total area of some 3.5 million hectares.

Sorghum *(Sorghum bicolor)*
Probably a native of North-East Africa, sorghum is now grown in most parts of the world as a source of grain, fodder, sugar and fibre. While some parts of Southern Europe grow it as a grain crop, interest has recently developed in growing sweet sorghum in Europe for the sugar syrups obtainable from its stems. These have been fermented to produce ethanol or methane. At the same time, fibrous sorghum varieties constitute an alternative source of cellulose for the paper and pulp industries. In 1999, 562 Gt were produced in the EU from an area of over 93,000 hectares. The producer Countries are Italy, France, Greece and Spain.

Miscanthus *(Miscanthus spp.)*
This tropical grass has been grown in Europe as an ornamental plant for several years. Thanks to their fast rate of growth, some species can be grown as a source of biomass in the warmer zones of Europe. Two species in particular, *M. sinensis* e *M. sacchariflorus*, are under investigation in many European Countries as a source of fuels for power stations and fibre for the paper industry.

Reed Canary-Grass *(Phalaris arundinacea)*
Found in the temperate regions of Europe, Asia and North America, where it is grown as a forage crop. The Swedes are currently investigating the possibility of growing it as a source of fibre and fuel.
The stems can reach 2 metres high and the leaves emerge in winter. Plantings may be used in a bed system to remove nutrients from waste water, as well to stabilise areas at risk of soil erosion.

Reeds *(Phragmites spp., Arundo spp.)*

Long used for textiles and musical instruments, common reed (*Phragmites communis*) is also a traditional thatching material. At present, its use as fuel is also being assessed. The stiff, smooth, hollow stems can reach a height of 1.5-3 metres. Giant reed (*Arundo donax*) is similar in form but much taller (up to 6 metres). *Arundo* is generally found in warmer, dryer habitats than *Phragmites* and has been indicated as a prolific source of biomass which could produce an annual 20-25 t/ha of dry matter for use as fibre or conversion into energy.

Cordgrass *(Spartina spp.)*
A perennial grass native to Western Europe, Africa and North America, has long, narrow leaves and stiff, erect stems which may be 130 cm tall in some species. Some experimental plantings in Britain have produced 15-20 t/ha dry matter a year. Since *Spartina* grows well in poor soil it is being considered as a possible source of biomass in less fertile zones.

Arable energy coppice – willow, poplar *(salix spp., Populus spp.)*
The coppiced branches of willow and poplar regenerate so fast that they represent a continuously and rapidly renewable source of biomass energy. In the past, both have been planted as ornamental shade or shelter trees, for firewood and for construction purposes. Today research is concentrated on ways of improving coppice management techniques in order to obtain a source of young wood for energy production.
Some eucalyptus species may represent possible alternatives for Southern Europe while birch is more suitable for growth in the far North. Fig. 13 shows the wide distribution of willow and poplar in Europe.

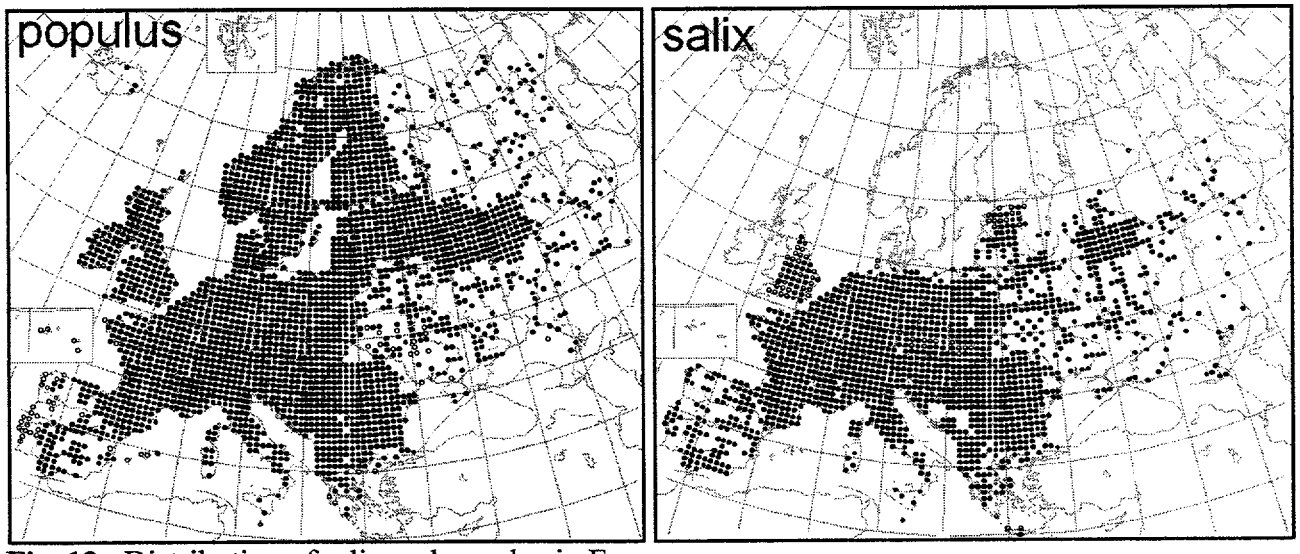


Fig. 13 - Distribution of salix and populus in Europe.
(Source: Botanical Museum, Finnish Museum of Natural History)

Ethiopian Mustard *(Brassica carinata)*
This is believed to be a natural hybrid that has only recently been investigated as a plant that might be grown in Europe. Its use as a source of biomass for biofuels is currently being evaluated. This mustard crop is currently grown in Denmark, France and Germany which produced nearly 6 Gt from an area of over 4,000 hectares in 1999.

BIOMASS FROM MUNICIPAL SOLID WASTE

Municipal solid waste (MSW) could be a major source of raw material for the production of ethanol. The paper, woody waste and organic matter in MSW contain cellulose and sugars that can be hydrolysed and fermented simultaneously into ethanol.

Almost 50% of all MSW is domestic in origin, the remaining 50% deriving from industrial, commercial and building activities. Upstream of differentiated collection processes, the composition of MSW varies from place to place: generally the largest fraction is paper (20-40%) followed by garden wastes (10-20%), plastics, glass, metals and other materials.

The management of municipal solid waste is summarised in Fig. 14.

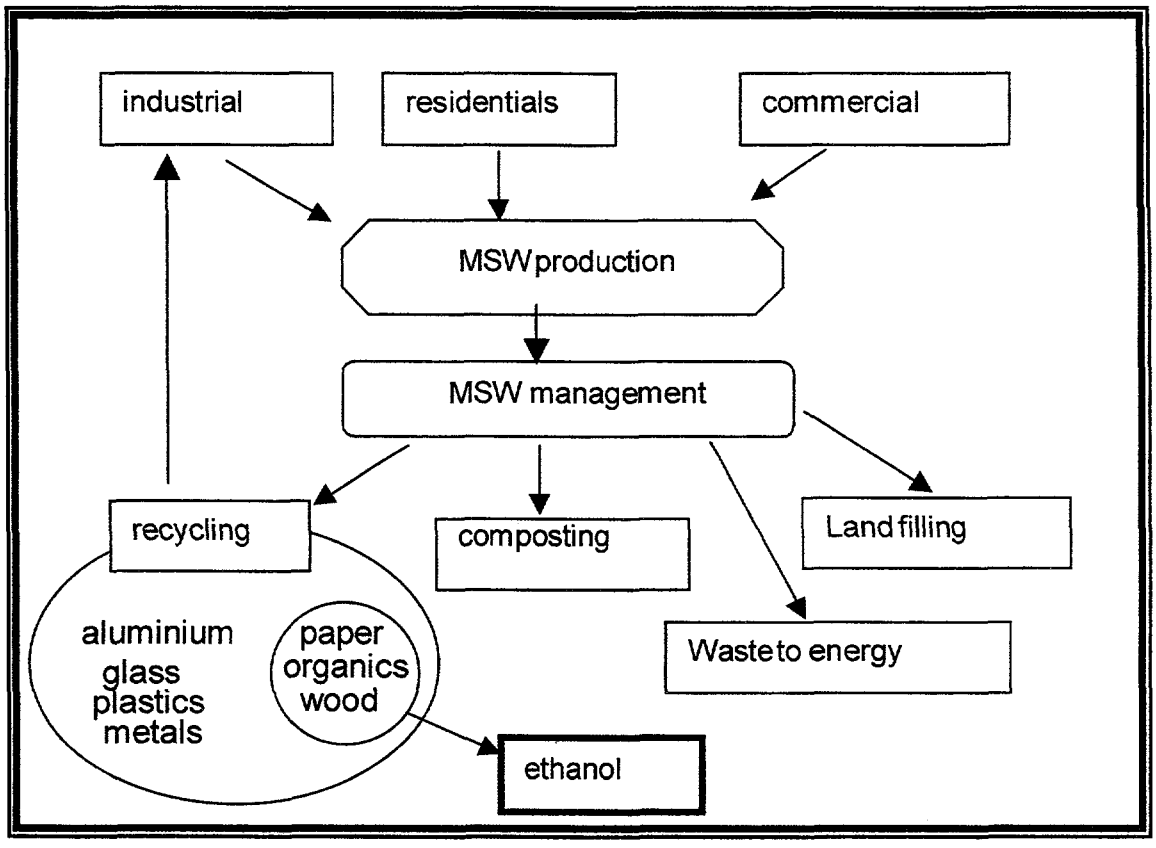


Fig. 14 -Municipal solid Waste management.

Waste recycling policies in EU Countries will give rise to ever-increasing quantities of biomass sorted out of MSW, which could be used in sectors like biofuel production. In Italy, where the annual production of MSW comes to 26 million tonnes, the Ronchi Decree has set a target of obtaining 35% sorted material from the total volume of MSW by early 2003.

Tab. 14 lists outputs of municipal solid wastes in the various EU Countries.

The ethanol obtainable from 1 kg dry solid waste that can be calculated on the basis of average waste composition (Tab. 15) and it is about 200 grams.

Tab. 14 - MSW production in EU.

	MSW total production in 1997 Ton x 1000
Austria	4,110
Belgium	5,028
Denmark	2,864
Finland	2,100
France	34,700
Germany	43,486
Greece	3,000
Ireland	1,325
Italy	26,605
Luxembourg	193
Netherlands	8,726
Portugal	3,500
Spain	15,307
Sweden	3,200
United Kingdom	35,000
TOTAL UE	**189,144**

(Source: European Energy Agency, 1998)

Tab. 15 - Ethanol potentially available from MSW.

	composition	*amount*	*Composition,%*		*amount*	**Ethanol***
MSW **1 kg** (d.m.)	paper	300g	cellulose hemicellulose	60 15	180g 45g	127g
	garden wastes	200g	cellulose hemicellulose	30 30	60g 60g	68g
	food wastes(15%)	150g	cellulose sugars	20 10	30g 15g	15g 8g
					TOTAL	**218g**

* *calculated from the processes: 1) cellulose □ glucose □ ethanol; 2) hemicellulose≅xylane □ xylose □ ethanol*
(SOURCE: Energy Information Administration, New South Wales)

TECHNOLOGIES OF ETHANOL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

Lignocellulosic biomasss is one of the virtually inexhaustible and easily available renewable sources of energy. In particular ethanol that can be produced from this source, is fast becoming a serious alternative to fossil fuels.

A wide spectrum of ethanol applications has been evaluated since the 1970s. It is a non-fossil transportation fuel, which contributes little, if any, to the net carbon dioxide in the atmosphere during production and use. In addition, it has a high-octane power and can be used in diesel fuel. Some recent issues address the employment of ethanol in ETBE (ethyl tertiary butyl ether) to replace the MTBE (methyl tertiary butyl ether) recently forbidden in California for suspected cancerogenic effects.

Many sources of lignocellulosic biomass, such as agricultural residues, forestry residues, pulp and paper waste, and municipal solid waste, are abundant and can be converted to ethanol. Woody and herbaceous energy crops can also be used as renewable resources for ethanol production.

In order to get ethanol from lignocellulosic biomass, its carbohydrates must be broken down into their monomeric sugars (*hydrolysis*) and then fermented using appropriate microorganisms. This can be achieved in two ways: *chemical hydrolysis* and enzymatic *hydrolysis*. The second procedure needs suitable and effective feedstock pretreatments in order to enhance the susceptibility of lignocellulosics to the enzymatic action.

BIOMASS CHEMICAL HYDROLYSIS

Concentrated acid hydrolysis

This process is based on the hydrolysis of cellulose using concentrated acid (10-30%) to get sugars at near theoretical yields. Sulphuric, hydrocloric and hydrofluoric acids are the ones mainly used. The Tennessee Valley Authority (TVA) began developing technology for converting cellulosic feedstocks to fuel ethanol in the 1950s (Barrier *et al.*, 1985; Boder *et al.*, 1988). Fig. 15 shows a flow diagram of the process (Broder *et al.*, 1995).

The process consists mainly of two stages. During the first phase, the biomass feedstock is mixed with 10% sulphuric acid and heated for about two hours to hydrolyse the five-carbon sugars of the hemicellulose. In fact the hydrolysis of hemicellulose requires a *medium* less acid than that required for cellulose. The suspension is then filtered in order to separate the solubilised C_5 and C_6 sugars from the solid residue (mainly lignin and cellulose). Following appropriate neutralization, the solution is sent to the fermentation unit. The solid residue is pretreated with concentrated acid to facilitate the next cellulose hydrolysis step. It is in fact mixed with 30% sulphuric acid and then heated to reduce the solvent volume and obtain a final acid concentration of 70%. The final step involves the conveyance of this suspension in hot water to a hydrolytic tank where an acid concentration of almost 10% is reached. The suspension is then heated at 100°C for almost two hours. This step completes the cellulose hydrolysis while avoiding any undesirable sugar degradation. After the solution is heated to obtain the total hydrolysis of the cellulose, it is filtered and the final liquid solution contains 10% acid and 10% glucose. This is neutralised with $Ca(OH)_2$ and after the gypsum $CaSO_4 \cdot 2H_2O$ is removed, the sugars are sent to the fermentation unit. The solid lignin residue is burnt for power production. Whole cellulose hydrolysis is accomplished in two steps: the concentrated acid (70%) disrupts the cristalline structure of the cellulose by cleaving the hydrogen bonds between the cellulose chains; the hydrolysis step is responsible for the hydrolytic reaction of the isolated cellulose chains.

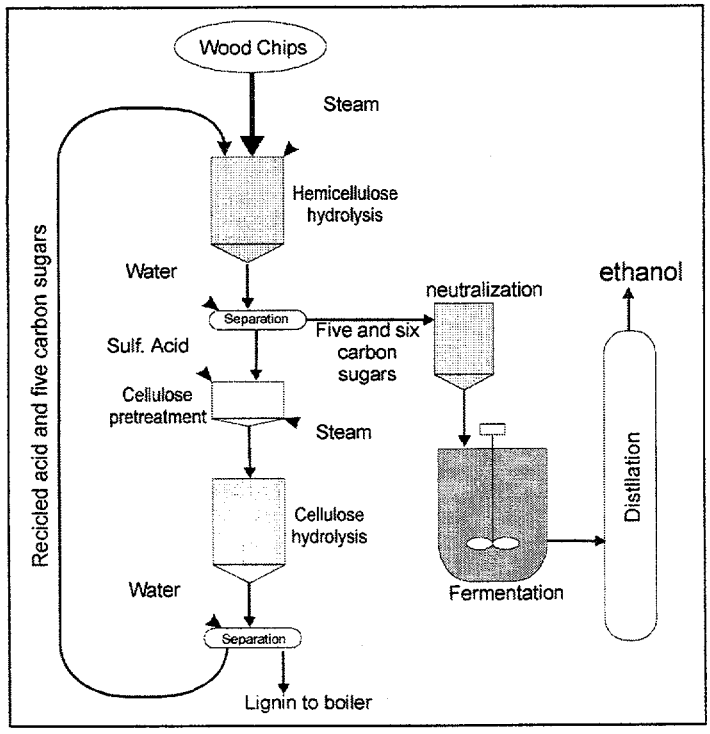


Fig. 19 – Scheme of the concentrated acid hydrolysis process *(Source: TVA).*

An important component of TVA's current program is a project to develop complete "biomass refinery systems" which convert feedstocks including crops such as alfalfa, and crop residues such as corn stover, as raw materials for multiple products (food, feed, energy, and chemicals). Cellulose and hemicellulose hydrolysis conversion efficiency relevant to different biomass feedstocks processed with concentrated acid is reported in Tab. 26.

Table 26 - Hydrolysis conversion efficiency of the TVA process (Broder *et al.*, 1990).

	Hemicellulose to xylose	**Cellulose to glucose**
	Conversion efficiency, %	*Conversion efficiency, %*
Alfalfa stem	96	88
Corn stover	92	90
Sugarcane bagasse	90	86
Oak wood	88	79

The ethanol yields from the glucose and xylose fermentations were almost 95% and 60% respectively.

A critical step in this process is the acid recovery and reconcentration. The development of cost-effective technologies to improve acid separation and recovery represents the way to open a new market for this process. Membrane separation is the most promising technology (Springfield *et al.*, 1999).

Two companies in the United States, Arkenol and Masada, are currently working with DOE (the US Department of Energy) and NREL (National Renewable Energy Laboratories) to market this technology by taking advantage of niche opportunities involving the use of biomass as means of reducing waste disposal or solving other environmental problems. In 1999, Arkenol planned to build a new biomass-to-ethanol plant near Sacramento using rice straw as a feedstock (Biofuels News, 1999). The other group is the Masada Resource Group, based in Alabama, which has

planned to set up a $30 million waste disposal/recycling facility in Middletown, New York. Besides the recycling of plastics, glass, metal, and waste paper, the plant would also use fermentation techniques to turn wastepaper and other cellulose materials into ethanol.
Arkenol's process shows some variations from the concentrated hydrolysis described above (Yancey *et al.*, 1997). In particular, de-crystallization involves adding 70%-77% sulphuric acid to biomass previously dried to 10% moisture. Acid is added at a ratio of 1.25:1 (acid: cellulose+hemicellulose), and the temperature is kept below 50°C. The solution is then diluted to an acid concentration of 20%-30% and heated at 100°C for an hour to hydrolyse the cellulose.

Dilute acid hydrolysis
In this hydrolytic treatment the hemicellulose and cellulose fractions of the biomass are recovered during two subsequent steps. The Fig. 16 reports a schematic flow diagram of the dilute acid hydrolysis (Border *et al.*, 1995).

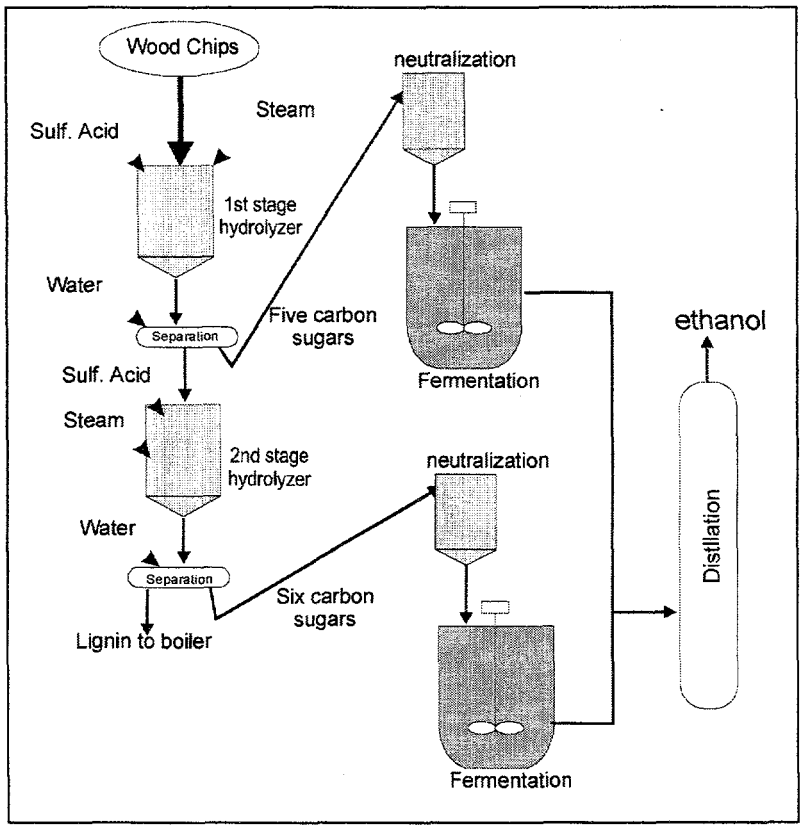


Fig. 16 - Diagram flow of a two stage dilute acid hydrolysis (Border *et al.*, 1995).

The first stage is conducted under mild conditions to hydrolyze the hemicellulose (typical process parameters from TVA are: 0.5% H_2SO_4, 160 °C), while the second stage is optimized to hydrolyze the more resistant cellulose fraction (typical process parameters from TVA are: 2% H_2SO_4, 200°C). After neutralization, the recovered liquid hydrolyzates are fermented into ethanol. Residual cellulose and lignin from the hydrolysis reactors are usually employed to power the boilers for electricity or steam production.
Some facilities have been planned in Jennings, Louisiana to convert hemicellulose sugars from molasses into ethanol using this technology. They will be built by the BC International Corporation (BCI) (Biofuels 1999).
Some of the earliest processes using dilute sulphuric acid produced 24% of ethanol theoretical yield (Larsson *et al.*, 1999). Through the use of percolation-type reactors (Scholler process), the ethanol yield was increased to 55-64%. The Scholler process was economically optimized in the US ("Madison process") where ethanol outputs of up to 60% of the theoretical yield were

reported. Several research projects have been devoted by NREL to the identification of the optimal conditions for maximum sugar and ethanol yields from this process (Nguyen *et al.*, 1999). Tab. 17 summarises the range of the investigated temperatures, residence times and acid concentrations.

Tab. 17 - Soluble sugar yields from mixed softwood obtained with dilute acid hydrolysis. The yields are expressed as percentages of theoretical values (Nguyen *et al.*, 1999).

Dilute acid conditions	T,°C	H_2SO_4 %	t, min.	Glucose yield, %	Xylose yield,%
first stage	200	0.4	5	49.9	63.5
second stage	215	0.7	3		
first stage	190	0.7	3	50.1	70.8
second stage	215	0.4	3		

Depending on the available hexose sugars, ethanol yields varied from 74 to 89% of the theoretical value.

Various reactor designs have been evaluated: percolation reactor, progressive batch/percolation reactor, counter-current and co-current reactors (Lee *et al.*, 2000).

Torget and his co-workers (Torget *et al* 2000) reported that whatever reactor is employed, the glucose yield from this process does not usually exceed 65-70%, which means the process is economically unfeasible. Several authors have proposed different explanations for the low yields obtainable with this technology. It could, in particular, be due to some degradation phenomena involving the fermentable sugars or to the formation during hydrolysis of cellulose oligomers resistent to fermentation. A recent approach employed by NREL has demonstrated that this yield could be raised to 85 % by changing the reactor configuration (Torget *et al.*, 2000). In this regard, promising results have been obtained using a *bench-scale shrinking – bed percolation* reactor in which an internal spring compresses the biomass as the reaction progresses. Though promising, this reactor needs further investigation to test the economic feasibility of scaling it up.

Dilute-acid hydrolyzates from lignocellulose are, to a varying degree, inhibitory to yeast. In particular, Taherzadeh (Taherzadeh *et al.*, 2000) found that the hydrolyzates contained between 1.4 and 5 g/l of furfural and between 2.4 and 6.5 g/l of HMF (5-hydroxy methyl furfural). For this reason a great research effort was devoted to finding the proper reactor configuration to ensure low sugar degradation and maximum recovery (Lee *et al.*, 2000). It seems (from theoretical analyses) that the countercurrent configuration should guarantee minimum sugar degradation thanks to the fact that the sugars travel a relatively short distance in the reactor before being washed out. A research team at the NREL has recently significantly advanced acid hydrolysis technology by experimentally proving that sugar yields higher than 95% from hemicellulose and 85% from cellulose are attainable in a multiple percolation reactor system simulating a countercurrent process (Torget *et al.*, 1997).

BIOMASS ENZYMATIC HYDROLYSIS

Under the condition of suitable pretreatment, enzymatic hydrolysis can ensure higher hydrolysis yields of polysaccharides than chemical hydrolysis. However the process suffers the disadvantage of the high costs of enzyme production (*vide infra*). Economical production of cellulase enzyme is the key for feasible bioethanol production from lignocellulosics using an enzyme-based process. Lignocellulosic material is a very complex substrate and for this reason it is required a mixture of effective enzymes capable of cleaving the different bonds inside the polymeric chain of the cellulose. The ability and, at the same time, the limitations of various enzyme mixtures to completely hydrolyze the structural polysaccharides of plant cell walls has been the focus of considerable research efforts over the years. The key to producing highly active cellulase inexpensively is a combination of critical factors: improved enzyme quality, extended enzyme lifetime, increased enzyme productivity and yield, and reduced costs of growth *medium* and substrate.

Cellulase enzymes are proteins with molecular weights ranging from 30,000 to 60,000 AMU and a typical ellipsoid arrangement with dimensions from min. 30 to max. 200 Å (Fat *et al.,* 1987). They are able to break the cellulose chain into its monomeric sugar constituents. The internal surface area of wood, which can be considered typical for a biomass, is very large but only 20% of the pore volume is accessible to cellulase-sized molecules. Thus, biomass pretreatment is necessary in order to increase the accessible surface area; this can be achieved by thinning the lignin-hemicellulose sheath surrounding the cellulose and by reducing the cellulose polymerization and crystallinity. Its final advantage is that it reduces the amount of hydrolytic enzymes needed, which still has a major impact on process costs. Although several improvements in biotechnology have been reflected in a reduction of the enzyme cost, it still accounts for over 40% of the total costs (Fan *et al.,* 1987; Philippidis *et al.,* 1997 ; Gregg *et al.,* 1998).
Basically, the pretreatments employed can be physical, thermal, chemical and biological depending on the action mechanism applied to the substrate. Sometimes a combination of two or more of these is taken into consideration with a view to producing synergetic effects.

Physical pretreatments
There are two categories of physical pretreatments, mechanical and non-mechanical (Fan *et al.,* 1982). The first class includes those procedures like *milling* and *grinding* in which the reduction in the substrate size is accomplished by an increase of the surface-to-volume ratio thus making cellulose more accessible to hydrolysis. Although *ball milling* is effective in reducing the cellulose crystallinity (McMillan, 1994), it often requires an impracticable treatment time (up to 7 days) and high processing costs to obtain any significant improvement in the saccharification percentage (Fan *et al.,* 1982).
The non-mechanical pretreatments provide a combination of mechanical forces with powerful external forces. This class includes pretreatments like *irradiation*, and *high pressure steaming*, which decompose lignocellulose. *Gamma irradiation* (50-200 kGy), which is considered a physical treatment, has been used to decrease the cell-wall constituents in some agricultural residues and to increase their digestibility (Al-Masri *et al.,* 1999). Although this treatment ensures an appreciable degree of hydrolysis it requires a long treatment time and a significant energy input (Fan *et al.,* 1982). For this reason it is not economically viable.

Steam-explosion

It is currently considered one of the most effective and costly physicochemical pretreatments for biomass (Zimbardi *et al.*, 2000, Ballesteros *et al.*, 2000). The process provides the use of saturated water steam at high temperature and pressure to cleave the chemical bonds between lignin, cellulose and hemicellulose. Biomass is chopped to an appropriate size and fed into a high-pressure reactor where it remains for a preselected time. At the end of the selected time, the treated biomass is expelled through a valve and the material literally explodes into a flash tank. The exploded biomass and volatile stream are recovered. The effect of this process on the biomass structure can be easily explained by the fact that the explosive decompression at the orifice causes the "flashing off" of the liquid water in the cellular structure of the substrate. The Fig. 17 shows the steam explosion reactor operating in the batch modality. The process cleaves off the acetyl groups from the hemicellulose fraction so providing an acidic *medium* conducive to hydrolysis. The combined mechanical and hydrolytic effect of the steam explosion treatment produces defiberization and cell disruption. In particular, after water extraction it leads to a physical separation of the biomass residues into its principal components (hemicellulose, cellulose and lignin) to an extent, which depends on the *severity* of the adopted treatment (Ibrahim *et al.*, 1999). For this reason steam-explosion makes the cellulose surface more easily accessible to enzymes and ensures a better hydrolysis of the cellulose component without any other treatment. The severity of the conditions is quantified by a semi-empirical parameter called R_0 that combines treatment time and temperature according to the following equation (Abatzoglou *et al.*, 1992):

$$R_0 = t \cdot \exp[(T-100)/14.75]$$

Where t is the time in minutes and T is the temperature in degrees Celsius.

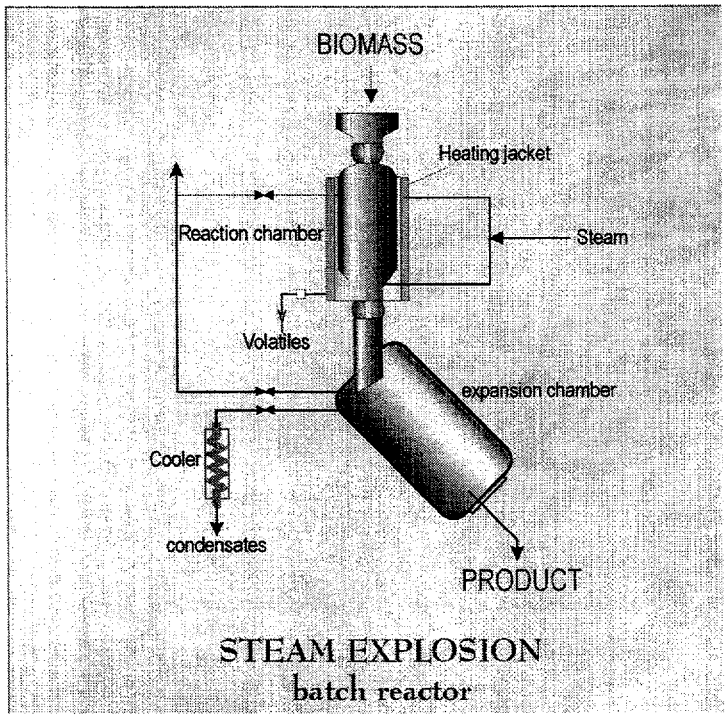
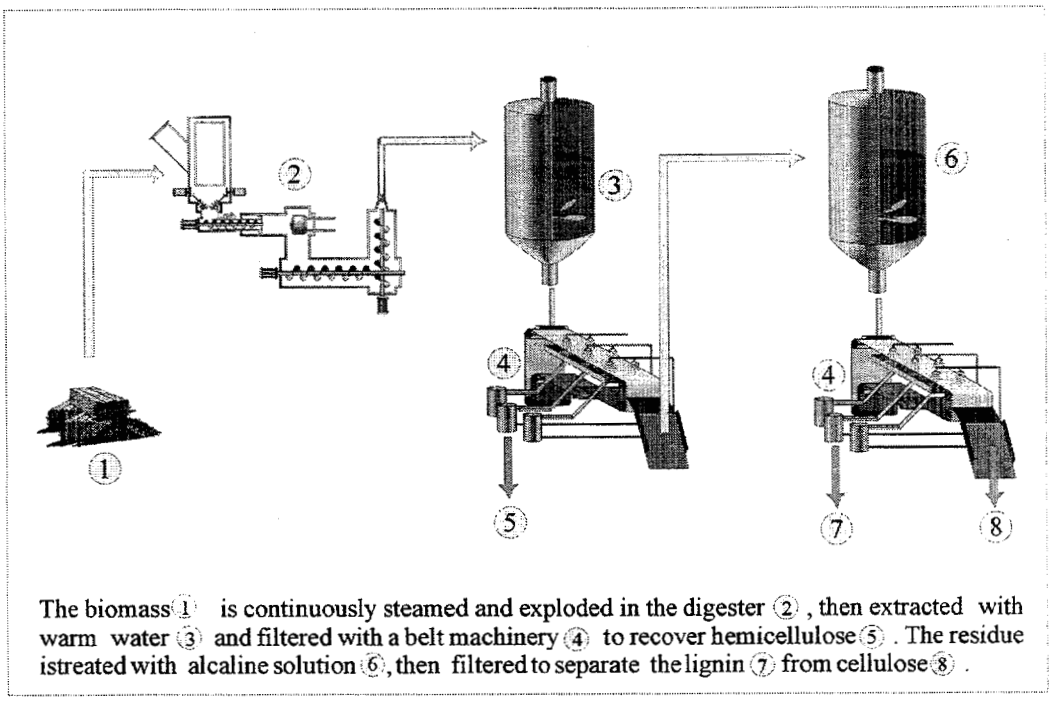


Fig. 17 - Steam explosion reactor operating in the batch modality.

Steam explosion fractionation has already proven successful on a wide range of substrates. The steaming times are relatively short (seconds to minutes), the steam pressure usually ranges from 1.5 to 4Mpa while the temperatures are in the 180-230°C range. This technique is reported in the literature as effective on a wide variety of biomass substrates: wood chips, macroalga, sugarcane

bagasse, oil palm cake and fiber, wheat straw, hemp, corn sillage, and bananagrass (Turn *et al.*, 1998). More recently some authors have investigated the application of the steam explosion technique to the segregation of the organic fraction from municipal solid waste (MSW) in order to recover a significant amount of cellulose and hemicellulose that can then be fermented into ethanol (Ballesteros *et al.*, 1998). The experimental results obtained show that the enzymatic saccharification of 10% (w/v) domestic organic waste produces yields of up to 81% using an enzymatic mixture of Celluclast and Novozyme (15 FPU/$g_{substrate}$ and 12.5 IU/$g_{substrate}$ respectively) at 50°C for 72 h.

Only two research centres in Europe have been involved in extending the lignocellulosic biomass conversion technology to the pilot scale. The first was a continuous industrial plant with a capacity of 2-4 t/h located in Souston, France (Ropars *et al.*, 1992; Ballerini *et al.*, 1994). This facility was built by a joint venture named Ascaf (Association pour le developpement des carburants par la fermentation) between AFME (Agence française pour la maitrise de l'energie) and IFP (Istitut francais du petrole). A wide range of biomasses was tested in this plant ranging from agro-residues (wheat straw, maize stalks *etc.*) to wood (poplar and pine). The second is a pilot scale plant and is located in Italy at the ENEA Research Centre at Rotondella. The facility can process up to 300 Kg of biomass per hour. Both the French and Italian facilities were built on the basis of a Canadian patent acquired from Staketech. The Fig. 18 shows the schematic diagram relevant to the steam explosion continuos plant operating in the Southern Italy.



The biomass (1) is continuously steamed and exploded in the digester (2), then extracted with warm water (3) and filtered with a belt machinery (4) to recover hemicellulose (5). The residue istreated with alcaline solution (6), then filtered to separate the lignin (7) from cellulose (8).

Fig. 18 – Scheme of the ENEA's steam explosion continuous plant: besides the steam reactor, also the extraction sections are displayed.

Since 1995, another facility has been built in Eastern Switzerland by a new biotech company, *2B AG* (Grass *et al.*, 2000). The 2B AG technology is similar to steam explosion pretreatment, providing steam treatment of the biomass and then enzymatic saccharification of the separated cellulosic fibers. The company has developed a continuous pretreatment unit with a capacity of almost 4t per hour. The maximum design pressure is 9 bar, with automated pressure control. Saturated steam is injected into this "cooker" at two different places. Residence time is controlled via a variable speed drive. This unit has been in operation since 1998. To date, the facility has processed several hundred tons of fresh grass at a throughput of almost 4t/h fresh grass. Operating pressure is typically 3-5 bar. The pre-treatment of 1 ton of dry clover/grass

matter yields the following products: 200-250 l ethanol, 150-300 Kg protein concentrate and 200-300 Kg fibers (data reported by 2B AG).
As already mentioned, the effectiveness of steam explosion pretreatment depends on the severity of the pretreatment regime adopted. However, under too severe pretreatment conditions carbohydrates and lignin are likely to form degradation products that inhibit the fermentation process. On the other hand, excessively mild conditions might not trigger the defiberization process so that enzymatic yields during hydrolysis would be low.

In order to make pre-treatment more effective, especially in the case of soft and hardwood, in some case the biomass was chemically impregnated before entering the reactor. This allowed for good total sugar recovery even at mild severity parameters. *Steam explosion using sulphur dioxide* (SO_2) as an acid catalyst improves the accessibility of the water-insoluble, cellulose-rich, component by enzymes, thereby allowing the release of the hemicellulose during the water phase with low sugar degradation. The oxidation of SO_2 to H_2SO_4 supplies the acidic *medium* favourable to plant carbohydrate hydrolysis (Shevchenko *et al.*, 2000). Although these two reagents should interact with the biomass in a similar reaction pattern, it was found that impregnation with SO_2 results in a more effective hydrolysis than the one with H_2SO_4 (Eklund *et al.*, 1995). This finding could be explained by the fact that the gaseous SO_2 penetrates the biomass fibres better than H_2SO_4 thus ensuring a stronger action. Some authors (Wu *et al.*, 1999, Shevchenko *et al.*, 2000) have explored pretreatment conditions in order to establish the best compromise between high enzymatic hydrolysis (achieved at high severity parameters) and good recovery of the hemicellulose components in the form of monomeric sugars (achieved under low severity conditions). Wu *et al.* performed some tests on steam pretreated Douglas Fir heartwood and soapwood chips. They found that after 72 hours of low severity pretreatment (logRo 3.45) the hydrolysis yield reached 24.2 %, while higher severity (logRo 4.21) produced an enzymatic yield of 97.7% (with a low recovery of hemicellulose sugars at just under 37.5%). As well as the residence time and the temperature of the pretreatment, the SO_2 level also affects the total sugar recovery (Stenberg *et al.*, 1998). Usually the concentrations employed are in the 1-6% (w/w) dry matter range. Pretreatment with 4.5% SO_2 followed by a *medium* severity treatment (logRo 3.45) provides a final hydrolysis yield of 86.6% with a C_5 component recovery of 64.7%.
More recently, other acid catalysts were tested in catalysed steam explosion pre-treatment (Tsuda *et al.*, 1998; Aoyama *et al.*, 1999). The catalysts tested were Lewis acids such as $AlCl_3$, $Al_2(SO_4)_3$, $FeCl_3$, inorganic salts such as NH_4Cl, $(NH_4)_2SO_4$, organic acid (acetic acid) and organic anhydrides like maleic anhydride, succinic anhydride, phtalic anhydride. The acid behaviour of the NH_4Cl and $(NH_4)_2SO_4$ solutions is due to the evaporation of NH_3 caused by the high temperatures of the treatment. Tsuda *et al.* tested these catalysts on bamboo grass culms whose composition is similar to that of hardwood. The Tab. 18 shows some experimental results obtained by these authors with steamed bamboo grass culms followed by hot-water extraction.

As can be inferred from the data reported in Tab. 18, acid catalysis significantly improves the enzymatic yield. With the exception of organic acid and anhydride for which a relatively large amount of catalyst is required for any enhancement in enzymatic yields, a significant increase can be observed even at low doses of loaded catalyst. Further discussions about the synergetic effect of combining chemical pre-treatment with steam explosion are reported in the paragraph concerning the chemical pretreatments.

Tab. 18 - Effect of the use of some acid catalysts on the enzymatic hydrolysis* of steam pre-treated bamboo grass culms (Tsuda *et al.*, 1998).

	Added amount mmol/Kg	**Enzymatic hydrolysis yield** (%) from the extracted residue
Uncatalyzed	0	60.7
$AlCl_3$	20	100
$Al_2(SO_4)_3$	10	97.5
$FeCl_3$	5	100
NH_4Cl	20	99.8
$(NH_4)_2SO_4$	10	100
CH_3CO_2H	30	89.9
Maleic anhydride	30	93.2
Succinic anhydride	20	67.8
Phtalic anhydride	30	97.0

**The hydrolysis was carried out at 40°C for 48 hours. The reported yields are based on the polysaccharides in the steam-extracted residues.*

The physical and chemical pre-treatments described above are based on the use of water, steam alone, or steam with small amounts of acids and are known as prehydrolysis, autohydrolysis, steaming, or steam explosion. When high-pressure oxygen or air is also injected into the reactor, the process is called *wet oxidation* (Schmidt *et al.*, 1998; Ahring *et al.*, 1999). This pretratment provides a hexose rich cellulose fraction (95-100% theoretical yield) and a pentose rich hemicellulose fraction (60% theoretical yield), and degrades lignin into low molecular weight phenolic compounds. Usually the process involves adding oxygen (3-12 bar) to the suspension of biomass in Na_2CO_3(2-6,5 g/l) and then heating at 150-200°C for about 15 minutes. The advantage of this pretreatment over steam explosion is its lower production of inhibiting agents. However the process requires the additional use of oxygen and alkali, which raise the cost of the pre-treatment.

Ammonia and carbon dioxide explosion

Another biomass pretreatment using a combined chemical and physical action is the *ammonia fiber explosion (AFEX)* technique in which biomass is treated with ammonia at a moderate temperature (300K) and high pressure (1.24 MPa) for about 30 minutes (Dale *et al.*, 1996; Vlasenko *et al.*, 1997). Finally, a valve is opened causing a sharp pressure drop, which literally explodes the fibrous biomass. The ammonia evaporates and up to 99% can be recovered and reused. The treatment effectively disrupts non-woody biomass such as bagasse and newspaper but it is not effective on non-softwood biomass (Hespell *et al.*, 1997). The sugar yields are near theoretical for the nonwoody biomass (Dale *et al.*, 1996) but no more than 50% of the theoretical yield for poplar (Belkacemi *et al.*, 1998; Ogier *et al.*, 1999).

More recently some efforts have been devoted to improving this kind of biomass pre-treatment by substituting ammonia with supercritical *carbon dioxide* (Zheng *et al.*, 1998). The resulting process should involve lower costs than the AFEX pre-treatment. In this technique the gas is in its *supercritical state* (it is compressed at a temperature above its critical point to a liquid-like density). The advantage of this technique resides in the fact that the supercritical fluid possesses

both the properties of a gas (easy mass transfer) and a liquid (solvating power). Moreover, it does not leave any residual material on the treated substrate, which could affect the subsequent steps. The experimental results obtained by Zheng *et al.* on substrate like recycled paper mix show that an increase in pressure from 7.6 to 20.7 MPa facilitates the penetration of carbon dioxide molecules into the crystalline structure thus increasing the hydrolysis yields. The obtained hydrolysis yield of biomass CO_2-exploded (at 20.7 MPa and 35°C) and subsequent hydrolysis (0.1% w/v of enzyme liquor, for 24 h) was 72.6%. The authors also tested the effectiveness of other gases like nitrogen and helium but carbon dioxide appeared to be the most effective.

Chemical pre-treatments

Many chemical pretreatments have been tested for the cleavage of the lignin sheet and the reduction of the crystalline content of cellulose before the enzymatic hydrolysis. Although several of these methods have been proven to be effective, they have the great disadvantage of requiring plants constructed of materials capable of resisting severe chemical conditions. The chemical agents tested for the purpose of biomass delignification are alkalis, acids, gases, oxidizing agents, cellulose solvent, and extraction and swelling agents.

The *Alkali pretreatment* is the most frequently used to increase the digestibility of some lignocellulose substrates. This treatment was developed as a pulping process to produce high strength, long fiber paper products (Zanuttini *et al.,* 1998). It was also trialed on straw and other non-wood materials, which still constitute the major fiber source for paper production (Lawther *et al.,* 1996). Several biomass substrates were subjected to this treatment and responded differently. Dilute NaOH pretreatment is somewhat more effective on straws than on hardwood owing, in part, to the lower lignin content of straws (McMillan, 1994). This treatment produces an increase in substrate digestibility thanks to the *swelling effect* of the Na^+ from the NaOH due to the counterionic action of the Na^+ on the carboxylic groups around the lignocellulosic backbone. The conditions usually employed in this pretreatment provide a concentration of 8 to 12% NaOH weight/dry matter, a residence time of 30-60 minutes and a temperature of 80-120°C. (Ogier *et al.,* 1999, Aiello *et al.,* 1996). The treatment has the disadvantage that the cost of the chemicals is high so that subsequent recycling procedures are required in order to recover the caustic soda. To date, this recovery procedure still involves prohibitive costs (Son *et al.,* 2000; Novalic *et al.,* 1998).

Sometimes, NaOH treatment takes the form of an *oxidative treatment* in an *alkaline peroxide medium* (Sun *et al.,* 2000; Curreli *et al.,* 1997). Hydrogen peroxide delignification of agricultural residues is strongly pH-dependent, with the optimum pH range for the dissociation of H_2O_2 between 11.5-11.6. At this pH range, the suggested mechanism for lignin oxidation provides the formation of chemical species (radicals) that are strongly reactive. The chemical reactions, which generate the active species, are reported below:

$$H_2O_2 + OH^- = H_2O + HOO^-$$
$$H_2O_2 + HOO^- = HO\bullet + O_2^{-}\bullet + H_2O$$

The OH• is responsible for the lignin oxidation. Krishna *et al.* (1998) obtained promising results by pretreating sugar cane leaves with alkaline hydrogen peroxide. A saccharification conversion yield of 92 % was achieved by hydrolysing the pretreated substrate at 50 °C and pH 4.5 using *Trichoderma reseei* cellulase (40 $FPU/g_{substrate}$) in 48 h.

Curreli *et al.* (1997) experimented a new mild alkaline/oxidative pretreatment of wheat straw prior to enzymatic hydrolysis that starts with an alkaline (1% NaOH for 24h) step to solubilise the hemicellulose and prepare the substrate, then a second alkaline/oxidative step (1% NaOH and 0.3% H_2O_2 for 24h) solubilizes and oxidises the lignin into low polluting compounds. The overall process was carried out at a low temperature (25-40°C) using a low concentration of

chemicals, resulting in relatively low cost and waste liquor containing only a trace of pollutant derived from the lignin.
Among the *oxidising agents* for biomass pretreatment *peracetic acid* has been recognized as a powerful, highly selective disrupter of the lignin structure. It opens the aromatic rings in lignin generating dicarboxylic acids and their lactones. The effects of this pre-treatment were tested at both high and low temperatures.
Texeira *et al.,* (1999) were the first to investigate a low temperature pretreatment approach. They compared a set of pretreatments with different peracetic acid concentrations with a combined pretreatment involving a preliminary soft alkaline soaking followed by the peracetic oxidation. In this last case, the first step serves to reduce the amount of peracetic acid to be employed in the second step. They found that a mixture of 6% NaOH and 15% peracetic acid was the optimal ratio from poplar and sugarcane bagasse samples to obtain a cellulose hydrolysis of 80-90%. Similar results were obtained using 60% peracetic acid alone. This procedure offers the advantage of not requiring an expensive reactor, and energy input as well as avoiding carbohydrate loss and the formation of inhibitors. However the use of peracetic acid significantly affects pre-treatment costs.

Both alkali and oxidative pre-treatment have potential disadvantages. Strong alkali, in fact, solubilizes and partially degrades hemicellulose into saccharinic acids, which are poorly utilized by microorganisms in the fermentation phase. Oxidative degradation of lignin can lead to the accumulation of phenolic monomers and oligomers, which are potentially inhibitors. A greater selectivity in the delignification of cellulosic feedstock can be achieved by a dilute alkali pretreatment in solvents for lignin (Sewalt *et al.,* 1997, M. G. Papatheofanous *et al.,* 1995). This technique is often referred to in the literature as *organosolv*. Some recent applications (Sewalt *et al.,* 1997) show that extensive delignification can be achieved by treating various grasses with a solution of 50% ethanol and 0.2M NaOH, by this treatment they achieved an enzymatic hydrolysis up to 78%.

In the previous section we have described *dilute sulphuric acid* as one treatment to hydrolyse the cellulose. However, due to its low hydrolysis yields, this procedure has recently been used mainly as a mean of pre-treatment.
It has been demonstrated that pretreatment effectively dissolves the hemicellulose and increases the enzymatic digestibility of cellulose. High reaction rates (compared with enzymatic processes), low acid consumption, and the low cost of sulphuric acid (compared with base-catalysed pretreatments) are some of the advantages of dilute-sulphuric acid pretreatment (Esteghlalian *et al.,* 1997). A dilute-acid pretreatment plant would not require an acid-recovery system, which seems essential for the concentrated acid process. The pretreatment conditions usually provide impregnation with approximately 1% (w/w) H_2SO_4 and then incubation at 140-160°C for a period ranging from several minutes to an hour (Dien *et al.,* 1999). One of the major problems associated with dilute acid hydrolysis of lignocellulosic biomass remains the poor fermentability of the hydrolysates produced (Tucker *et al.,* 2000). In lignocellulosic hydrolysates, the concentration of sugars as well as the concentration of by-products depends on the hydrolysis conditions. Severe conditions can degrade sugars to furfural, levulinic acid and formic acid while only a minor part of the lignin is degraded, resulting in a range of aromatics.
Recently at NREL the effectiveness of combining chemical pretreatment by dilute- sulphuric acid with steam explosion has been tested on whole tree chips (Nguyen *et al.,* 2000). In particular, these authors compared the pretreatment via single- and two-stage dilute-sulphuric acid treatment before delivery to the steam-explosion reactor. While in single-stage pretreatment ($logR_o$ 3.13-3.75; sulphuric acid concentration in wt%: 0.80-2.38) wood chips were treated at a wide range of severities, in the two-stage pretreatment, the first stage was carried out at low severity ($logR_o$ 2.96; sulphuric acid concentration in wt%: 2.66) to maximize hemicellulose recovery. In the latter case, the solubilized sugars were then recovered from the solid extract by

washing with water. The water-insoluble solids from the first-stage prehydrolysate were impregnated with dilute sulphuric acid (2.5% w/w), then steam treated under more severe conditions ($logR_o$ 3.54) to hydrolyze a portion of the remaining cellulose into glucose and to improve enzyme digestibility. The overall sugar yield from two-stage dilute-acid pretreatment was approximately 10% higher, and *the net enzyme requirement was reduced by about 50%.* The same authors have also proved the effectiveness of this pretreatment also on a feedstock of mixed municipal solid waste. The digestibility of water-washed, pretreated MSW was 90% in batch enzymatic hydrolysis at 66 $FPU/g_{cellulose}$.

Apart from the chemical methods for biomass pretreatment, some authors (Sreenath *et al.,* 1999) have proposed *liquid hot water* (*LHW*) pre-treatment which merely washes the biomass with flowing preheated water at 220 °C for 2 min. The water was held at sufficient pressure to ensure that it remained in the liquid phase. The technique was successfully applied to the pretreatment of Alfalfa *Medicago sativa* fibre and the enzymatic hydrolysis tests carried out on the separated solid substrate at 50 °C show yields near the theoretical.

Biological pretreatment
In recent years, progress in bioengineering has led to the development of microorganisms capable of attacking the lignin in the biomass. The ligninolitic enzymes could be potentially useful in several areas of industrial forestry processes, pulp manufacture, waste management and by-product utilization thanks to the broad substrate specificities (Kuhad *et al.,* 1997).
Some authors (Palmieri *et al.,* 1997-2000) have conducted studies of the enzymes produced by the basidiomycete *Pleurotus ostreatus*. This micelium produces at least five different phenol oxidases (laccases) one of which is secreted in larger amounts. These enzymes are homologous proteins characterised by different specificity towards the substituted phenols. Another well-known basidiomycete fungus already proven effective in delignification is *Phanerochaete chrysosporium* and the ligninolytic enzymes, which can be isolated from it (lignin peroxidase and manganese peroxidase. (Thompson, 1998; Brumer 1999; Reddy, 2000) Although it looks like a very promising field, a lot of work will have to be done in order to create the basis for development from the lab to the pilot scale.

THE ENZYMES USED FOR THE HYDROLISYS OF LIGNOCELLULOSIC BIOMASS

The enzyme mix responsible for the hydrolysis of cellulose to glucose is called cellulase and is constituted of *endo* 1,4 β-glucanase and *exo* 1,4 β-glucanase, β-glucosidase). The Fig. 19 schematically shows the process by which the enzymes cleave the cellulose structure.

The mechanism of cellulose hydrolysis is quite complex. Several studies seem to agree in identifying two components in the enzyme structure that plays a complementary role in the hydrolysis process: namely, *C1* factor and *Cx* factor (Fan *et al* 1987). The C1 factor is responsible for the conversion of the cellulose into its linear more reactive form while the Cx factor (endoglucanase and exoglucanase) breaks down the linear chains into soluble oligosaccharides, usually cellobiose. This is finally transformed into glucose by the *β-glucosidase* (Fig. 19).

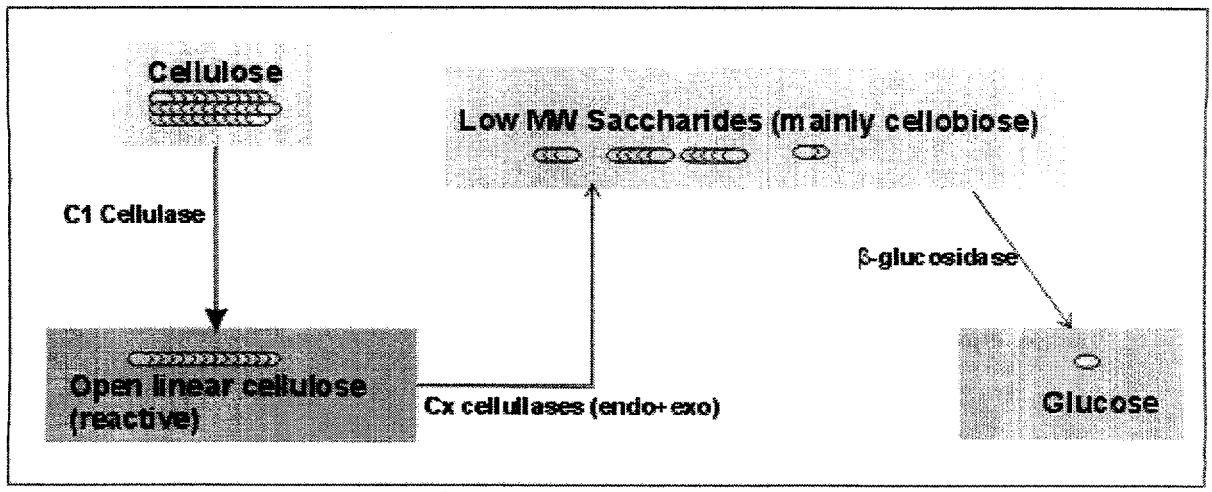


Fig. 19 - Mechanism for the enzymatic cellulose hydrolysis.

The general classification of the enzymes is based on the site where the reaction takes place: *intracellular* (or *cell-associated enzymes)* and *extracellular* enzymes. The main function of the extracellular enzymes is to transform the substrate by acting on the cell mass constituents into the external *medium*; by contrast the intracellular enzymes require that the substrate be diffused through the cell mass before it can be converted. The endoglucanase and exoglucanase activities of cellulases are extracellular whereas β-glucosidase activity is cell-associated (cellobiose must diffuse through the cell mass to be converted into glucose). The action site of cellulolytic enzymes is important in the design of hydrolytic systems because it can affect the reaction time.

Hydrolytic enzymes are synthesised by fungi and bacteria in the presence of carbon sources, typically soluble and insoluble carbohydrates (*e.g.* cellulose, cellobiose, lactose *etc.*). The microorganisms produce the enzymes, which serve to convert the carbon source into glucose, which is easily accessible to the microorganism metabolism. The major cellulase-producing microorganisms that have been used at pilot or industrial scale are the fungi *Trichoderma Reesei* and *Aspergillus niger*, and the bacterium *Penicilium funiculosum*. Other fungal cellulase producers include *Trichoderma viride*, *Trichoderma lignorum*, *Trichoderma koningii*, *Penicillium spp.*, *Fusarium spp.*, *Aspergillus spp.* The amount of hydrolytic enzymes produced depends on the nitrogen source, the type and concentration of the carbon source, pH and temperature. The enzymes produced in this way are called *adaptive* (or *inducible*) because their synthesis has been triggered by an inducing agent (there also exist *constitutive* enzymes which are always present in the cell). Thus cellobiose, for example, usually in concentrations not higher than 1% acts as inducing agent for *Trichoderma reesei* .
Very promising results have been obtained with the *Clostridium thermocellum* bacterium that shows interesting properties because its methabolism is both able to produce cellulase enzymes and subsequently to ferment the glucose obtained. In this way, a direct bioconversion of the cellulose to ethanol is possible. The relevant process is called Direct Microbial Conversion (DMC) to account of this direct conversion. The main problem of this process is the low tolerance of this bacterial strain to certain levels of ethanol (Chandrakant *et al.* 1998). However, some authors (Rani *et al.* 1998) recently found two *Clostridium thermocellum* strains, namely SS21 and SS22, which are able to ferment pure crystalline cellulose with a maximun ethanol yield ($g_{ethanol}/g_{substrate}$) of 0.37 and 0.35 at a low substrate concentration (8g/l).

Enzyme activity
Enzyme activity is influenced by several parameters (Hettenhaus *et al.*, 1997). The most important is the *temperature* since an increase of 20-30°C can introduce a 3 to 5 fold improvement. However, the enzymes are proteins and high temperatures can cause an

undesirable denaturation. In addition the specificity of the enzyme activity plays an important role. In fact the enzyme acts by binding to the solid substrate of cellulose. If the fermentation *medium* contains other solid products (*e.g.* lignin), an enzyme loss could occur owing to parallel adsorption onto lignin. The specificity of the enzyme interaction site could reduce the overall enzyme activity by up to 50%.
Another issue to be resolved is *product inhibition*. Enzyme activity can be strongly influenced by the presence of certain levels of cellobiose, glucose or products such as furfural and organic acid coming from the pretreatment step. Genetic modification can be introduced into the enzyme biochemistry in order to raise the inhibition threshold, this can double the enzyme activity.
Finally, the extent of the substrate decristallynization to provide to the enzyme a bigger cellulose surface should be considered. Since cellulose hydrolysis occurs more easily in acid conditions, an enzymatic mixture containing enzymes able to operate low pH without the risk of denaturation would be an important factor not to be forgotten. Finally a further improvement in this respect can be obtained by increasing the number of enzyme domains able to bind onto the cellulose backbone.
In order to compare the efficiency of enzyme mixtures in hydrolysing a given cellulosic substrate, an index has been established: the Filter Paper Units (FPU) and the International Units (IU). The most widely used substrates for the determination of total cellulolytic activity include filter paper, microcrystalline cellulose, cotton fibers and soluble carboxymethyl cellulose. Each method usually involves measuring the reducing sugars (glucose) formed during the enzymatic hydrolysis of the substrate. Filter paper hydrolysis, the so-called Mandels-Weber method (Mandels *et al.*, 1976), has generally been accepted for this purpose. In particular, the method measures cellulase activity in units of micromoles of reducing sugars liberated per minute under standard assay conditions.

FORMATION OF INHIBITORS

Ideally, the bioconversion of wood-to-ethanol process should convert all of the wood carbohydrates in the feedstock to ethanol. However, both dilute acid hydrolysis and the conditions usually adopted in the steam explosion pre-treatment led to at least partial degradation of the hemicellulose-derived sugars and significant solubilisation and transformation of lignin related chemicals (Ando *et al.*,1986). To better utilise all the available carbohydrates and to minimise the production of potentially inhibitory products it is possible to recover more than 80% of the original hemicellulose in the water-soluble stream after steam explosion (Douglas fir wood chips) (Boussaid *et al.*, 1997). However, one of the downsides to optimising pretreatment solely for maximum hemicellulose sugar recovery could be that lignin-cellulose complex is not readily fractionated and that insoluble, cellulosic components would not be efficiently hydrolysed by cellulolytic enzymes. Conditions can be selected that should provide maximum solubilisation and recovery of the hemicellulose component (low severity), optimum enzymatic hydrolysis of the water insoluble cellulosic component (high severity), and a compromise between the two conditions (*medium* severity). To combine the residence time and temperature during hydrolysis or pretreatment into a single reaction ordinate, the severity factor (R_o) was introduced. A third parameter, the environmental pH, into the above equation was introduced to describe the combined severity (CS) (Chum *et al.*, 1990):

$$CS = \log R_o - pH$$

Prior to the enzymatic hydrolysis, the cellulose structure is made available to the hydrolysing enzymes by pretreating the material. Wood can be pretreated using high-temperature steam which solubilises the hemicellulose. In order to improve the recovery of hemicellulose-derived sugars, the wood can be impregnated with SO_2 or H_2SO_4 prior to steam explosion (Clark and

Mackie, 1984). The advantage of SO_2 and H_2SO_4 impregnation of willow prior to steam explosion at 200 to 210°C was shown in (Eklund *et al.*, 1995).
When the CS is increased beyond the value, which generated maximal mannose and glucose concentrations, hemicellulose sugars and glucose are broken down. The decrease in the concentration of fermentable sugars coincided with the formation of furfural and hydroxymethylfurfural (HMF), which, in turn, are degraded to levulinic and formic acids when the CS is further increased. The fermentability drastically decreases around the value of CS where furfural and HMF start to accumulate. To obtain both high yields of fermentable sugars and high fermentability CS should be kept at about 3, as reported in (Palmqvist and Hahn-Hägerdal, 2000-b).
A schematic diagram of the most important reactions occurring during the hydrolysis of lignocellulosic substrate is reported in Fig. 20. The furan derivatives and phenolic compounds will react further to form some polymeric material.

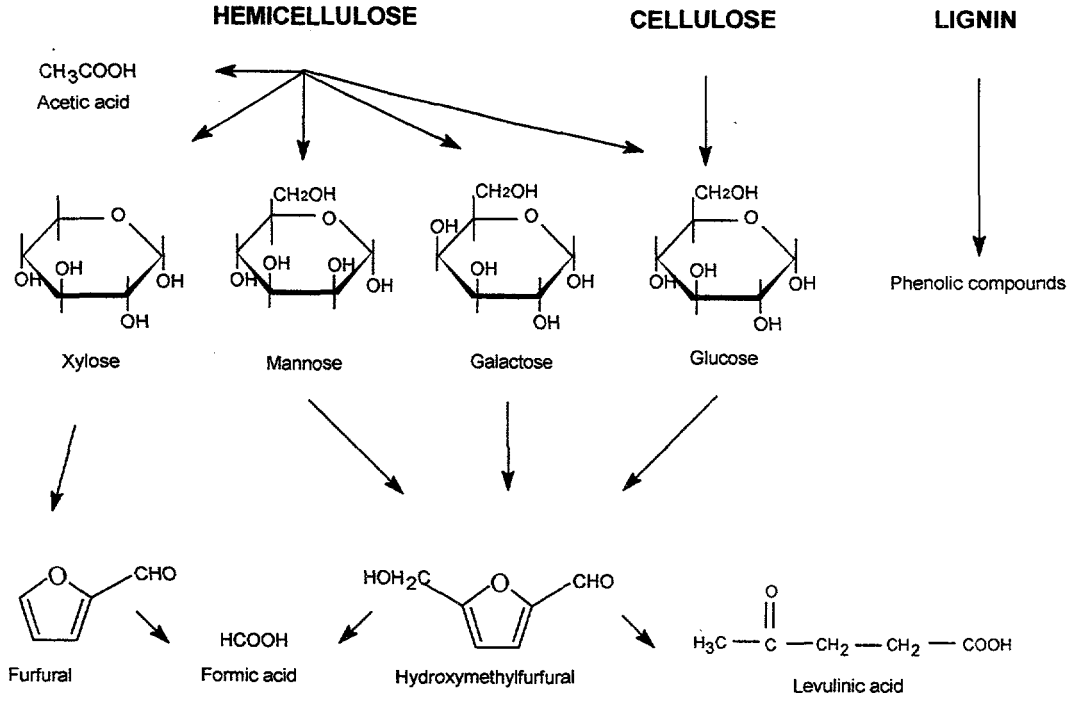


Fig. 20 - Reactions occurring during hydrolysis of lignocellulosic materials.

The inhibitors can come from several sources; *e.g.* equipment, carbohydrate decomposition, lignin decomposition, wood extractives and their decomposition (Leonard and Hajny, 1945). Hibbert's ketones have been detected in the hydrolysate of pine (Clark and Mackie, 1984). Vanillic acid and vanillin, formed by the degradation of the guaicylpropane units of lignin, have been detected in hydrolysate from willow (Jonsson *et al.*, 1998), poplar (Ando *et al.*, 1986), red oak (Tran and Chambers, 1986), and pine (Clark and Mackie, 1984). In hardwood hydrolysates, syringaldehyde and syringic acid, formed in the degradation of syringyl propane units, have been reported (Jonsson *et al.*,1998; Tran and Chambers, 1985). Hydroquinone has been identified in hydrolysates of willow (Jonsson *et al.*,). 4-Hydroxybenzoic acidic constitutes a large fraction of lignin-derived compounds in hydrolysates from hardwood poplar (Ando *et al.*, 1986) and willow (Jonsson *et al.*, 1998). Extractives, capronic acid, caprilic acid, pelargonic acid, and palmitic acid have been reported in dilute hydrolysate of red oak (Tran and Chambers, 1985). This substances are detrimental to the overall process as sugar degradation products such as furfural and hydroxymethylfurfural as well as phenolics from lignin degradation have been

shown to inhibit fermentation of the wood-derived sugars to ethanol (Olsson and Hahn-Hägerdal, 1996).
Different methods have been proposed to deal with the problem of toxic compounds. Basically, four different approaches can be distinguished: 1) avoiding the formation of the inhibitors during hydrolysis, 2) detoxification of the hydrolysates before fermentation, 3) development of inhibitor-tolerant strains, and 4) *in situ* detoxification based on bioconversion of the toxic compounds. These methods allow alleviating the inhibition caused by the degradation products. However, in addition to losing part of the initially available carbohydrate, these procedures (physical-chemical detoxification techniques, such as neutralisation, and fungal treatment) add an extra step to an already complex process (Olsson and Hahn-Hägerdal, 1996; Palmqvist *et al.*, 1997).

Inhibitor identification and formation processes
In the pretreatment of lignocellulosic biomass, which is often performed at acidic pH and temperatures around 200°C, water-soluble compounds that are inhibitory to microorganisms are released (Palmqvist *et al.*, 1997). Equally, in the pretreatment with SO_2 at 205°C the hemicellulose is hydrolysed. The solubilized sugars, mostly pentoses, together with generated soluble inhibitors must be washed with water prior to the enzymatic hydrolysis of the cellulose fibers. To make a large-scale industrial process economically feasible, the sugars in this hydrolysate have to be utilized. The formation of inhibitory compounds was with accuracy observed since long time in wood hydrolysates derived from the softwood *Pinus radiata* (Clark and Mackie, 1984) and during the use of steamed hemicellulose as a substrate in microbial conversions (Buchert *et al.*, 1989).
Another restriction, which is laid on a large-scale process, is the necessity to recirculate process water. It was shown that a non-concentrated hydrolysate is only moderately inhibiting whereas a five-time concentration of nonvolatile components (see next chapter for the definition of nonvolatile compounds) results in almost complete inhibition of the ethanolic fermentation by *Saccharomyces c.* (Palmqvist *et al.*, 1996).
In addition to soluble mono- and oligosaccharides, the extraction of steam-exploded wood with lukewarm water (50°C) solubilizes low-molecular weight phenols. Evidences are especially reported for the treatment of poplar wood (Excoffier *et al.*, 1991). On the other hand, trihydroxybutyric acids (THBA) are obtained in the course of the purification of lignin; the formation of this last type of compounds, and more generally of carboxylic acids, during wet oxidation of cellulose, hemicellulose or carbohydrates at high temperature, have firstly been described by Mc Ginnis *et al.* (1984).
Most of the toxic compounds are formed in the hydrolysis with dilute acids by undesirable side reactions (Taherzadeh *et al.*, 2000). Enzymatic hydrolysis can represent a valid method to deal with the problem of toxic compounds, and a good alternative to the dilute-acid hydrolysis. Enzymatic hydrolysis, however, requires a chemical pretreatment to make the wood structure accessible to the enzymes. Pretreatment, although less severe than dilute-acid hydrolysis, gives rise most likely to the formation of some inhibitors.
The inhibitors can be schematically classified according to their chemical structure into organic acids, furans and phenolic compounds (Palmqvist *et al.*, 1999-b). Inhibitors of fermentation include: furan derivatives, such as furfural and 5-hydroxy-methyl-furfural (5-HMF); aliphatic acids, such as acetic acid, formic acid, and levulinic acid; and phenolic compounds (Larsson *et al.*, 1999-b). Furfural and 5-HMF are products of pentose and hexose degradation (Larsson *et al.*, 1999-a). The pentose sugars are primarily transformed to furfural while hexoses are transformed into hydroxymethylfurfural. Both pentoses and hexoses can also be transformed to aromatic compounds or condense to lignin-like products. This was confirmed by the results of an investigation dealing on *medium* consistency suspensions during the dilute acid hydrolysis of lignocellulosics (hardwood) in a plug flow reactor (Abatzoglou *et al.*, 1990). Furfural is produced by the Maillard reaction and occurs as a by-product during high temperature hydrolysis

of cellulosic matter (Navarro, 1994). Furfural and 5-HMF can be further broken down to formic and levulinic acid.

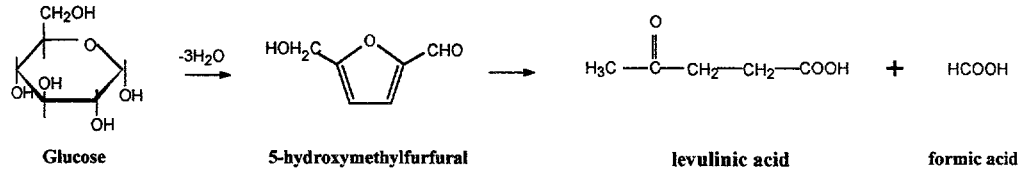


Fig. 21 - The formation of levulinic and formic acids during dilute acid hydrolysis of spruce.

In Fig. 21, the pathway of 5-HMF degradation forming levulinic acid and formic acid is reported (Larsson *et al.*, 1999-a). In addition, formic acid can be formed from furfural under acidic conditions at elevated temperature (Dunlop, 1948).
Acetic acid is liberated from hemicellulose during hydrolysis (Fan *et al.*, 1982; 1987). A minor part of lignin is degraded during the hydrolysis process, generating a wide range of aromatic compounds (Larsson *et al.*, 1999-a).
In addition to the products formed by decomposition of carbohydrates and lignin, and to compounds derived from wood extractives, another group of microbial inhibitors have been identified in wood hydrolysates: inhibitors derived from the metal or minerals in wood, soil or hydrolysis equipments (Frazer and Mc Caskey, 1989; Parajò *et al.*, 1997-b).
Some works report high recoveries from hardwoods such as willow (Eklund *et al.*, 1995) when SO_2 and H_2SO_4 impregnation of willow is performed prior to steam pretreatment. Considerably lower recoveries have generally been reported for softwoods, see the study on the influence of SO_2 impregnation and fractionation on product recovery and enzymatic hydrolysis of steam treated sprucewood (Schwald *et al.*, 1987). Hemicellulose sugar recovery decreased as the steam explosion severity increased as a result of the degradation of monomeric sugars, by dehydration and condensation reactions, which tend to occur at higher temperatures and longer cooking times. Sugar degradation products of the water-soluble fractions derived by an original 20% dry weight of Douglas fir chips is reported in Tab. 19. The pretreated materials, adjusted to a dry matter content of 20% (w/w) with deionized water, were filtered through Whatman microglass-fiber filter paper under suction (Boussaid *et al.*, 1999). The resulting filtrates were used for water-soluble hydrolysates characterisation and fermentation.

Tab. 19 - Sugar degradation products of the water-soluble fractions derived by an original 20% dry weight of Douglas fir chips (Boussaid *et al.*, 1999).

Severity factor (logRo)	Furfural (g/l)	HMF (g/l)
3.08	0.8	0.91
3.45	2.3	1.84
3.76	4.10	3.64

The fermentation of the water-soluble fractions preadjusted to pH 6.0 with NaOH and sterilised through a 0.45-µm Nylon membrane over a 48-h period with SSL-adapted strain of *Saccharomyces c.* indicates an inverse relationship between the severity factor and both sugar composition and ethanol production. Only the hexoses were used with galactose only utilised after most of the mannose and glucose were consumed. The highest ethanol concentration of (15 g/l) was obtained with the water-soluble fraction obtained after *medium*-severity pretreatment followed by the low-severity derived fraction (12 g/l). Another evidence of the effect of

pretreatment severity and inhibitor formation is reported in (Larsson *et al.,* 1999-a) The optimum CS for producing ethanol from dilute acid-hydrolysate spruce was between 2.9-3.1. This CS reduces to the lowest level the concentration of inhibitors in the hydrolysate. Furfural and HMF derived from sugar degradation, well-known fermentation inhibitors (Navarro, 1994; Olsson and Hahn-Hägerdal, 1996), could contribute to lower the initial ethanol production rate. Other wood inhibitory compounds such as extractives and lignin-derived phenolics, vanillin and syringyl aldehyde, are also known to be potent inhibitors of ethanol fermentation by *Saccharomyces c.* (Ando *et al.,* 1986; Delgenes *et al.,* 1996). However, despite the presence of these inhibitory compounds, both hydrolyses obtained at low and *medium* severity led to high ethanol yields (0.44 g of ethanol produced per gram of hexoses utilised).
Finally, it is interesting to point out that adsorption and inactivation of the cellulase system on lignin are limiting factors, as well as inhibition by small molecules produced during steam pretreatment. Removal of the lignin with diluted alkali enhances the cellulose enzymatic hydrolysis, due to the increase in accessible surface area. Furthermore, a sodium chlorite bleaching of the alkali insoluble steam exploded biomass, enhances the hydrolysis, due to the removal of the residual lignin, which has been found to be a strong inhibitor of β-glucosidases. However, this kind of post-treatment maybe not economically viable (Palmqvist *et al.,* 1996).
D-xylose from hemicellulose hydrolysis, at 5% concentration, results in ca. 13% inhibition of *A. niger* β-glucosidase activity (Dekker, 1986).

Toxicity: mechanisms and kinetics of inhibition
Both the rate of enzymatic hydrolysis and ethanol fermentation are affected by the presence of toxic compounds generated during either biomass pretreatment in the steam explosion process or in the hydrolysis step with low acid concentration. The lignin degradation products include a wide range of aromatic and polyaromatic compounds with a variety of substituents. It is likely that some of these aromatic substances inhibit both the hydrolysis and fermentation steps. It is also probable that the solubilisation of extractives in the pretreatment produces potent inhibitors in low concentration.
Trihydroxybutyric acids (THBA) and phenols are inhibitors of enzymatic hydrolysis (Excoffier *et al.,* 1991). The decrease of glucose yield can be explained by the inhibition of β-glucosidase both by THBA, even at low concentration, and by phenols. THBA can easily form lactones in water solution, and inhibitions of β-glucosidase by gluconolactones have been described (Dekker). Inhibition of different *Trichoderma* cellulases by p-cumaric and vanillin has been already reported (Martin and Blake, 1989). Inhibition of enzymatic hydrolysis of cellulose by furfural and hydroxymethylfurfural has never been demonstrated (Sinitsyn *et al.,* 1982). In the following Table 30, the data refer to the enzymatic hydrolysis of pure microcrystalline cellulose in the presence of two inhibitors contained in the water extractives of steam exploded poplar wood chips. *Trichoderma reesei* CL-847 was used as cellulase preparation. Partially in contrast, it is reported that phenol is not an inhibitor of cellulolytic enzymes but a number of substituted phenols, such as *o*-hydroxybenzylalcohol, are moderately inhibiting (Palmqvist *et al.,* 1996). The same Authors in their study on the effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis found that the substances with the greatest inhibitory effect on the enzymatic hydrolysis, carried out with Celluclast (from Novo Nordisk) were in the nonvolatile fraction. The nonvolatile fraction contains acetic acid and lignin-derived compounds. The nonvolatile fraction was prepared as follows. The pretreated biomass was filtered in a Larox filter-press unit at a pressure of 14 bar to 50% dry matter (DM). The filtrate was collected and evaporated into three liquid fractions using a Rotovapor operating at 230 mm Hg. The first fraction obtained, comprising approximately 10% of the original filtrate, contains the most volatile components. The remainder is evaporated until only 10% remained, and is rich in sugars and other nonvolatile compounds. The intermediate fraction is discarded.

Tab. 20 - Enzymatic hydrolysis of pure microcrystalline cellulose in the presence of two inhibitors contained in the water extractives of steam exploded poplar wood chips.

THBA, *g/l*	**Glucose**, *arb. unit*	**Cellobiose**, *arb. unit*	**TOTAL**
0	43	57	**100**
1	26	59	**85**
5	25	60	**85**
10	21	69	**90**
Phenols			
2	27	65	**92**
5	17	56	**73**
10	13	60	**73**

Holtzapple *et al.*,(1990) state that most of the confusion on the inhibition of *Thichoderma reseei* cellulase results because it is difficult to conduct conclusive experiments which show the type of inhibition. A second point of their analysis is also important especially with regards to the possibility of performing the simultaneous saccharification and fermentation and of conducting the enzymatic hydrolysis in the presence of solvents. Ethanol and butanol are noncompetitive inhibitors. In contrast, acetone is a slight activator of cellulase.

Another aspect of the toxicity problem is related to the process configuration. In an environmental sustainable process, the water streams have to be recirculated both to minimize the use of freshwater and the amount of wastewater (Palmqvist *et al.*, 1997). Due to recirculation there will be a build-up of inhibitory compounds at different stages in the process as shown in the study on the simulation of ethanol production processes based on enzymatic hydrolysis of lignocellulosic materials using ASPEN PLUS (Galbe and Zacchi, 1991). The computer simulation of ethanol production from lignocellulosic materials indicates that, in some process units, the concentrations of recirculated substances may be 5-20 times higher than those found without recirculation. In addition, the accumulation of nonvolatile substances is slightly higher than that of volatile substances in the hydrolysis and fermentation units.

In a simultaneous saccharification and fermentation process (SSF), the recirculation of enzymes and microorganisms can create also the need of sterility. When the fibrous material is thoroughly washed with water and stored at -18°C prior to hydrolysis and the flasks with fibrous material are autoclaved for 15 minutes at 121°C infections does not appear. After sterilisation of the substrate, *medium*, and flasks, the SSF could be performed under nonsterile conditions without any infection being observed after 72 hours. The transfer of enzyme and yeast to the SSF can be done without any particular precautions. In contrast, infection appears after about 2 days of SSF when the substrate, the SSF *medium*, or the flasks were not autoclaved prior to SSF. In conclusion, the *medium* and the substrate must be autoclaved prior to fermentation. In addition, softwood has been found to be more difficult to utilise than hardwood, because softwood is more resistant to hydrolysis (Gregg and Saddler, 1996; Stenberg *et al.* 2000,; Tengborg *et al.*, 1998). In the simultaneous saccharification and fermentation, yeast adaptation may be necessary to make it withstand inhibiting byproducts.

Studies on the effect of water-soluble inhibitors from steam-pretreated willow on ethanol fermentation have shown that an accumulation of non-volatile components present in the hydrolysate severely inhibits the ethanolic fermentation by *Saccharomyces c.* (Palmqvist *et al.*, 1996; Palmqvist *et al.*, 1997). The presence of compounds inhibitory to microorganisms, such as *Saccharomyces c.* (Ando *et al., 1986*) and *Candida guilliermondii* (Sanchez and Bautista, 1988)

in steam-pretreated wood has long be acknowlegded (Mes-Hartree and Saddler, 1983; Pampulha and Lauriero-Dias, 1989). However, general conclusions are difficult to be reached because lignocellulosic hydrolysates vary in their degree of inhibition, and different microorganisms have different inhibitor tolerances (Palmqvist and Hahn- Hägerdal, 2000-a). The fact that even different strains of *Saccharomyces c.* may vary in inhibitor tolerance has been illustrated in a comparison between the performance of *Saccharomyces c.* baker's yeast, and *Saccharomyces c.*, ATCC 96581, isolated from a plant for fermentation of spent sulphite liquor, SSL, (Palmqvist *et al.*, 1998).

Four major groups of inhibitors have been identified in lignocellulosic hydrolysates: acetic acid from the hemicellulose fraction, lignin-degradation products, sugar degradation products, and extract which are solubilized in the pretreatment (Olsson and Hahn- Hägerdal, 1996). These compounds act individually and possibly also synergistically. Usually, fermentation inhibitors in lignocellulosic hydrolysates can be divided into several groups depending on their origin, see Tab. 21.

Firstly, substances released during prehydrolysis and hydrolysis includes acetic acid and extractives. These latters comprise terpenes, alcohols, and aromatic compounds such as tannins, according to the results of Frazer and Mc Caskey (1989) on wood hydrolysate treatments for improved fermentation of wood sugars to 2,3-butanediol. Secondly, a group of inhibitors (furfural, 5-HMF, levulinic acid, formic acid, and humic substances) is produced as by-products in prehydrolysis and hydrolysis due to degradation of sugars. Thirdly, lignin degradation products include a wide range of aromatic and polyaromatic compounds with a variety of substituents. Fourthly, products from the fermentation process, such as ethanol, acetic acid, glycerol, and lactic acid, inhibit the microorganism. The influence of these compounds is especially evident in recirculating systems (Nishikawa *et al.*, 1988). Finally, metals released from the equipment and additives such as SO_2 will also inhibit fermentation as proven in the study on the reactions of *Saccharomyces c.* and *Zygosaccharomyces bailii* to sulfite (Pilkington and Rose).

Buchert *et al.*, (1990) found more than 60 compounds when a hemicellulose hydrolysate of steam-pretreated birchwood was analysed with capillary GC. The literature does not offer a clear picture of which inhibitors have the most pronounced effect, but acetic acid and lignin degradation products are suggested to be the inhibitoriest (Baugh *et al.*, 1988). Among the identified toxins, furfural, HMF, levulinic acid, acetic acid, formic acid, and various phenolic compounds originating from lignin (Chung and Lee, 1985) were the most studied. In regard to fermentation to produce ethanol, substancial research has been made in the investigation of individual toxins including furfural (Azhar *et al.*, 1981; Banerjee *et al.*, 1981-a), HMF and formic acid (Ingram *et al.*, 1955), and levulinic acid (del Rosario *et al.*, 1979).

Furfural and 5-HMF affect the growth and metabolism of microorganisms. Furfural is the inhibitoriest to fermentation by *Saccharomyces cerevisiae.* (Palmqvist *et al.*, 1996). The inhibiting concentration of furfural is 1.3-3.2 g/l although concentrations lower than 2 g/l were reported to have little effect on fermentation (Tran and Chambers, 1985; Wilson *et al.*, 1989).

In alcoholic fermentation, the inhibition is alleviated if high cell inocula are used. A high cell *inoculum* (10 g/l) alleviates their toxicity (Palmqvist *et al.*, 1996). This seems to be the case for furfural on ethanol production by *Saccharomyces c.* in batch culture. The substances give rise to a lag phase proportional to their concentration, *Saccharomyces c.* can metabolize furfural; after it has been consumed the inhibitory effect disappears (Boyer *et al.*, 1992). During fermentation, furfural is reduced by yeast cells to furfuryl alcohol (Palmqvist, 1998). Furfural reduction to furfuryl alcohol creates a lag phase in ethanol fermentation (Chung and Lee, 1985; Villa *et al.*, 1992)). The alcohol dehydrogenase of yeast cells is responsible for the reduction of furfural, which inhibits the respiration, to furfuryl alcohol; the growth rate is affected (Weigert *et al.*, 1988). Furfural creates only a lag-phase in the ethanol formation, but does not reduce the final ethanol yield (Chung and Lee) and is consumed more rapidly than 5-HMF.

Tab. 21 - Effects of inhibiting compounds on fermentation (Olsson and Hahn- Hägerdal, 1996).

Group of inhibitors	Inhibitor	Concentration (g/l)	Microorganism	Inhibition of growth, %	Inhibition of fermentation,%
Compounds released during pretreatment					
	Acetic acid	1.4	*Saccharomyces c.*		50%; pH 4.5
	Acetic acid	4.3	*S. cerevisiae*		50%; pH 5.5
	Acetic acid	8.0	*Pichia stipitis*		98%; pH 5.1
	Acetic acid	8.0	*P. stipitis*		25%; pH 6.5
Sugar degradation products					
	Furfural	1.0	*P. stipitis*	47%	71%
	5-hydroxymethyl furfural	3.0	*P. stipitis*	69%	90%
Lignin degradation products					
	Cinnamaldehyde	1.0	*S. cerevisiae*		100%
	p-hydroxybenzaldehyde	0.4	*Klebsiella pneumoniae*	68%	
	p-hydroxybenzaldehyde	1.0	*S. cerevisiae*		48%
	Syringaldehyde	0.5	*K. pneumoniae*	40%	
	Syringaldehyde	0.22	*P. stipitis*		72%
Fermentation products					
	Acetaldehyde	5.0	*S. cerevisiae*	80%	
	Ethanol	120	*S. cerevisiae*	100%	
	Formic acid	2.7	*S. cerevisiae*	80%	
	Lactic acid	38	*S. cerevisiae*	80%	
Remaining					
	Chromium	0.1	*Pachysolen tannophilus*		95%
	Copper	0.04	*P. tannophilus*		29%
	Iron	0.5	*P. tannophilus*		45%
	Nickel	0.05	*P. tannophilus*		92%

Dead cell count showed that the extent of dead cells was dependent on the initial furfural concentration. Analysis of furfural showed that it was depleted during the fermentation. The larger the initial cell number, the lower the death cell and the depletion time (Navarro, 1994).
Acetic acid is liberated from naturally occurring acetylated hemicellulose (Parajò *et al.,* 1997-a) and is released through the partial hydrolysis of hemicellulose. The inhibitory effect of weak acids is pH-dependent (Larsson *et al.,* 1999-b). Very high concentrations of weak acids may result in acidification of the cytoplasm and cell death. Acetic acid is inhibitory to yeasts in the range 0.5-9 g/l . Acetic acid is a well known food preservative that permeates the cell membrane when undissociated and then dissociates in the cytoplasm where the pH is almost neutral. The concentration of undissociated acids in lignocellulosic hydrolysates being very dependent on pH therefore, pH is a crucial variable during fermentation. The cell uses energy to pump out surplus of H^+ ions in order to maintain its intracellular pH. This eventually leads to cell death at high acetic acid concentration. The toxic effect is less pronounced at a higher pH when the acetic acid is dissociated and cannot permeate the cell wall. The inhibitory effect is thus related to the concentration of the undissociated form of the acetic acid, which is a function of pH and pKa. At pH 5.5 and 5.0 the concentration of undissociated acetic acid are in the range from 0.36 to 0.84 g/l . These levels would cause the fermentation rate of *Saccharomyces c.* to decrease to 75% and 50% of the fermentation rate without acetic acid respectively. Ethanolic fermentation by *Saccharomyces c.* was inhibited by nonvolatile compound fraction present in lignocellulosic hydrolysates (Palmqvist *et al.,* 1996) that contains acetic acid as well.
Two mechanisms have been proposed to explain the inhibitory effect of weak acids: uncoupling and intracellular anion accumulation (Palmqvist and Hahn- Hägerdal, 2000-b; Russell, 1992). According to the uncoupling theory, the drop in intracellular pH resulting from inflow of weak acids is neutralised by the action of the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis. Additional ATP must be generated in order to maintain the intracellular pH, and under anaerobic conditions this is achieved by increased ethanol production at the expense of biomass formation. At high acid concentration, the proton pumping capacity of the cell is exhausted, resulting in depletion of the ATP content, dissipation of the proton motive force, and acidification of the cytoplasm. According to the anion accumulation theory, the anionic form of the acid is captured in the cell and undissociated acid will diffuse into the cell until equilibrium is reached. Since the equilibrium concentration of the undissociated acid is a function of pH, the extent of intracellular anion accumulation will be a function of the pH gradient over the plasma membrane (Larsson *et al.,* 1999-b).
It is also reported (Larsson *et al.,* 1999-a) that acetic acid, formic acid, and levulinic acid up to 100 mmol/l increase the ethanol yield. Higher concentrations than this resulted in a decrease in ethanol yield. Formic acid may be inhibitorier than acetic acid and levulinic acid due to smaller size of molecule, facilitating its diffusion through the cell membrane and probably causing higher anion toxicity. Levulinic acid may be slightly inhibitorier than acetic acid due to greater lipophilicity.
Lignin-degradation products probably exert further inhibition, for instance p-hydroxybenzoic acid that, because of its lipophilicity, causes leakage of H^+ into the cell.
The fact that inhibition decreases considerably by specifically removing the phenolic compounds with the enzyme laccase (Jonsson *et al., 1998*), implies that the phenolic compounds are major inhibitors in lignocellulosic hydrolysates (Palmqvist and Hahn- Hägerdal, 2000-a). Supporting this is the observation that a dilute-acid hydrolysate of spruce is considerably inhibitorier than a model fermentation containing the corresponding concentrations of weak acids, furfural and HMF, but no phenolic compounds (Larsson *et al.,* 1999-a).
The mechanism of inhibition by aromatic compounds has not yet been elucidated; however low molecular-mass phenolics are inhibitory (Larsson *et al.,* 1999-b). Of these compounds, the low molecular weight phenolics have been suggested to be the inhibitorier (Ando *et al.* 1986,; Clark and Mackie, 1984; Larsson *et al.,* 1999-a).

The most abundant aromatic monomers, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde exert a certain degree of inhibition on ethanol fermentation (Ando *et al.,* 1986; Palmqvist *et al.,* 1996). Cinnamaldehyde and cinnamic acid are more inhibiting, but much less abundant. Less-substituted phenolics are inhibitorier than vanillyl and syringyl derivatives as Nishikawa *et al. (1988)* have showed it in their study on the influence of lignin degradation products on xylose fermentation by *Klebsiella pneumoniae.*

Saccharomyces c. and *Zymomonas mobilis* growth was also tested (Delgenes *et al.,*) in the presence of furaldehyde (0.5-2 g/l), acetate (5-15 g/l), hydroxymethylfuraldehyde (1-5 g/l), vanillin (0.5-2 g/l), hydroxybenzaldehyde (0.5-1.5 g/l), and syringaldehyde (0.2-1.5 g/l). The results demonstrated that *Saccharomyces c.* was capable of being acclimated to high level of hydroxymethylfuraldehyde and vanillin. The result is in accord with those discussed in (Ando *et al.,* 1986; Azhar *et al.,* 1981). Acetate has the less toxic effect of the tested inhibitors for both strains. Considering the inhibiting effect of the tested model molecules on glucose fermentation, *Zymomonas mobilis* showed higher potential, except with hydroxybenzaldehyde, for both ethanol and cell biomass production than *Saccharomyces c.*, in accordance with the results of Fein *et al.* (1984-a).

Furaldehyde is assimilated by the two strains at a rate that increases with an increase in its initial concentration, in accordance with the results of (Sanchez and Bautista). Hydroxymethylfuraldehyde is consumed at a significantly lower rate particularly from *Saccharomyces c.*, which exhibits a lag period of 24 hours. No acetate consumption is observed. Syringaldehyde is assimilated during sugar fermentation except for *Zymomonas mobilis.* Only *Saccharomyces c.* has the ability to significantly consume high levels of hydroxybenzaldehyde. Vanillin is assimilated by both tested strains.

In conclusion, the inhibitors make detoxification necessary before it is possible to ferment the hydrolysates. The acetic acid cleaved from acetylated compounds in the wood, the furfural and HMF as breakdown products from pentose and hexose sugars, respectively (Sanchez and Bautista, 1988) and lignin degradation products affect ethanol yield and fermentation time. A correlation was found between the fermentation rate and the sum of furfural and HMF concentrations (Taherzadeh *et al.,* 1999). However, the observed correlation need not be an isolated effect of these compounds; instead, an interaction effect involving several inhibitors is most likely involved (Palmqvist *et al.,* 1999-b). If it is the case, removal of one, or two, of the involved compounds may be sufficient to yield a fermentable hydrolysate and to define the required detoxification step.

DETOXIFICATION AND ADAPTATION

Several methods of detoxification for the removal of inhibitors from lignocellulosic hydrolysates have been proven to increase their fermentability. Chemical, physical, and biological methods can be used to remove inhibitors prior to the fermentation (Larsson *et al.,* 1999-b; Olsson and Hahn- Hägerdal, 1996). The development of custom-made detoxification methods would be interesting as well as the modification of the prehydrolysis and hydrolysis processes to minimise the formation of the most potent inhibitors (Baugh and Mc Carty). Different detoxification methods have various effects on the hydrolysate, but in most instances the inhibitors are only partly removed (du Preez, 1994).

Different detoxification methods cannot be strictly compared when different lignocellulosic hydrolysates and different microorganisms have been used (Palmqvist and Hahn- Hägerdal, 2000-a). The choice of the detoxification method must be made after considering the composition of the hydrolysate and the type of raw biomass. For instance hardwood hydrolysates contain higher concentration of acetic acid and furfural than softwood hydrolysate (Taherzadeh *et al.,* 1997).

Detoxification increases the cost of a process. The detoxification cost can constitute 22% of ethanol production cost (Von Sivers *et al.,* 1994). As one of the dominating costs is the cost of the raw material (about 30%), this necessitates a high utilisation of the raw material and confirms the need to ferment both the hexoses and the pentoses. This is especially important for hardwoods, which contain a high concentration of pentoses (Ladisch *et al.,* 1993).
In the literature, the possibility of increasing the fermentability of the sugar solutions by various detoxification methods is widely discussed. Procedures for detoxification, such as ion exchange (Clark and Mackie, 1984), calcium hydroxide treatment (Leonard and Hajny, 1945) charcoal and molecular sieve adsorption (Tran and Chambers, 1985) and steam stripping (Yu *et al.,* 1987) have been tested at laboratory and for application in large-scale industrial processes. The Table 32 reports a list of the most suggested detoxification procedures and the main effect of detoxification.
The following discussion elucidates the different categories of procedures reported in the literature. Interaction between the effects of different inhibitors plays an important role. Acetic acid and furfural have been shown to interact antagonistically on growth, *i.e.,* the observed decrease in the specific growth rate in the presence of both compounds was greater than the sum of the decrease caused by the individual compounds (Palmqvist *et al.,* 1999-b). In addition, the importance of the detoxification and the optimization of the procedures may suffer in some cases of empiricism. Phenolic compounds have been suggested to exert a considerable inhibitory effect in the fermentation of lignocellulosic hydrolysates, the low molecular weight compounds being most toxic (Clark and Mackie, 1984; Buchert *et al.,* 1989). However, the mechanism of the inhibiting effect has not been elucidated, largely due to a lack of accurate qualitative and quantitative analysis.

Tab. 22 - List of suggested detoxification procedures.

Procedure	**Effect of detoxification**
Steam stripping	Removal of volatiles (furfural, phenols) including acetic acid
Neutralisation with CaO, NaOH, KOH; activated carbon; filtration	Reduction of acetic acid concentration
Neutralisation to pH 6.5 or overtitration to pH 10 with $Ca(OH)_2$, CaO, or KOH; removal of precipitate; H_2SO_4 to pH 6.5	Precipitation of acetate, heavy metals, furfurals, tannins, terpenes, phenolics
Ion exchange chromatography (cation exchange resin)	Removal of aromatic monomers and dimers
Ether extraction	Removal of furfural
Evaporation under vacuum	Removal of acetic acid
Ethylacetate extraction	Removal of lignin-degradation products
Molecular sieve	Partial removal of acetic acid, furfural, and soluble lignin
Mixed bed ion resins	Part. removal of acetic acid, furfural, and soluble
Anion/cation exchange resins	Part. remov. of acetic acid, removal of metal ions

Biological detoxification

Treatment with the enzymes peroxidase and laccase, obtained from the ligninolytic fungus *Trametes versicolor*, has been shown to increase the maximum ethanol productivity in hemicellulose hydrolysate of willow two to three times (Jonsson *et al.*,). The laccase treatment led to selective and virtually complete removal of phenolic monomers (2.6 g/l in the crude hydrolysate) and phenolic acids. The detoxifying mechanism was suggested to be oxidative polymerisation of low molecular weight phenolic compounds. The filamentous soft-rot fungus *Trichoderma reesei* degrades likely inhibitors in hemicellulose hydrolysates obtained after steam pretreatment of willow. Ethanol productivity and ethanol yield increase three and four times, respectively (Palmqvist *et al.*, 1997). The result was related to the removal of acetic acid, furfural and benzoic acid derivatives from the hydrolysate by the treatment with *Trichoderma reesei*.

Palmqvist *et al.* (1997) show that the inhibition could be totally eliminated by prefermenting the hemicellulose hydrolysate with the filamentous fungi *T. reesei*. At the same time, cellulolytic enzymes are produced and can be used for the enzymatic hydrolysis of the cellulosic fraction of the biomass. The ethanolic fermentation of the detoxified hydrolysates with *S. cerevisiae* results in as good and even better yields and productivities than in fermentation containing only glucose and nutrients. During 5 hours of fermentation at reference conditions (glucose and nutrients) the ethanol yield is 0.37, in the case of undetoxified hydrolysate is 0.11, and in the case of detoxified concentrated is 0.42. This result was attributed to the action of acetic acids that acts as an uncoupler on *S. cerevisiae* up to a concentration of about 5 g/l (Tran and Chambers, 1985).

Treatments with laccase (Larsson *et al.*, 1999-b; Jonsson *et al.*, 1998) and with *Trichoderma reesei* RUT C30 NRRL 11460 (Larsson *et al.*, 1999-b) were also tested for the detoxification of lignocellulosic hydrolysates. Treatment with laccase is performed at pH 5.5 (adjusted with 10% w/v NaOH) according to the procedure suggested in (Jonsson *et al.*,). In the treatment with the fungus the *inoculum* constitutes 10% v/v of the final volume. Control treatment are always performed along with the enzymatic treatment and the fungal fermentation by adding water instead of the enzyme solution and the *inoculum* to account for any changes in the composition of the hydrolysate during the treatment owing to factors other than the enzymatic activity and the fungal growth. A higher ethanol yield than in the reference fermentation was observed after laccase treatment. This could be owing to aliphatic acids, which are still present after detoxification. The filamentous fungus *T. reseei* improves the fermentability of dilute-acid hydrolysates even though the treatment does not affect the concentration of acetic acid. The presence of aromatic compounds does not decrease during the laccase treatment whereas an increase of large-sized materials and a decrease of small-sized material were observed. Therefore, the detoxifying mechanism was suggested to be oxidative polymerisation of low molecular weight phenolic compounds. In contrast, treatment with *T. reesei* results in a decrease of aromatic compounds, indicating that the mechanisms of detoxification are different.

Finally, a selective removal of acetic acid from hardwood-spent sulphite liquor using mutant yeast was tested by Schneider (1996).

Physical detoxification

This category of detoxification procedures includes extraction with solvents, evaporation of hydrolysates, steam stripping, ion exchange and exclusion, separation with addition of activated charcoal, or molecular sieves and electrodialysis.

Solvent extraction shows several interesting features (Cruz *et al.*, 1999), including: i) good fermentation performance achieved using solvent-detoxified media, ii) easy recovery of solvents, owing to their low boiling point, an aspect that favours the overall economic features of the process, and iii) easy recovery of solubilised lignin fractions, which might be used as cheap, renewable source of food antioxidants.

Ethers and ketones are among the most employed solvents for removing phenolics from water (Cruz *et al.*, 1999). Ethyl acetate and diethylether have been used for extracting low molecular weight phenolics from oak wood (Fernandez de Simon, *et al.*, 1996). Five organic solvents (chloroform, ethyl acetate, trichloroethylene, diethyl ether and hexane) have been tested in the experiments reported by Clark and Mackie (1984) and Leonard and Hajny (1945). Extraction with organic solvents is also reported in (Parajò *et al.*, 1997-b; Frazer and Mc Caskey, 1989; Fein *et al.*, 1984-b; Wilson *et al.*, 1989).

The solvent extractions procedures that are reported in the literature are very close each other.

In the work of Cruz *et al.* (1999) the hydrolysates and the selected solvent are contacted in 250 ml baffled Erlenmeyer flasks with orbital shaking (300 rpm) at constant temperature (in the range 10-40°C). pH is readjusted to 3 or 6.5 with $CaCO_3$ or to alkaline with $Ca(OH)_2$. Organic phase is vacuum evaporated at temperatures under 40°C, and the residual water is removed by freeze-drying. Extracted hydrolysates show an enhanced susceptibility towards fermentation in relation to unextracted hydrolysates. The best results correspond to hydrolysates at pH 3 extracted with hydrolysate/solvent volume ratio of 1:3.

Parajò *et al.* (1997-b) used the same hydrolysate/solvent volume ratio for solvent extraction of both neutralised and overlimed hydrolysates. 50 ml of hydolysates are treated with 150 ml of solvent. Chloroform, ethyl acetate, and trichloroethylene perform in a similar way in both neutralised and overlimed hydrolysates, decreasing the phenolic contents of the solutions by 28-50%.

After continuous, overnight, extraction of a strongly inhibiting spruce hydrolysate with diethyl ether at pH 2, the ethanol yield is comparable to the value in reference fermentation containing glucose and nutrients (Palmqvist and Hahn- Hägerdal, 2000-a). The ether extract contains acetic, formic, and levulinic acid, furfural, hydroxymethyl furfural and phenolic compounds. In agreement with this result, ethyl acetate extraction increases the ethanol yield in fermentation by *Pichia stipitis* from 0 to 93% of that obtained in reference fermentation due to the removal of acetic acid (56%) and complete depletion of furfural, vanillin, and 4-hydroxybenzoic acid (Wilson *et al.*, 1989) The low molecular weight phenolic compounds are suggested to be the most inhibiting compounds in the ethyl acetate extract.

However, after extraction of the inhibitory ether fraction of a dilute acid hydrolysate of spruce with water (three times), the water phase has again been found inhibitory in fermentation assays, showing that the major inhibitors are relatively soluble both in the aqueous and in the organic phase. When $NaHCO_3$ 0.5 M is used for extraction, in order to further fractionate the inhibitor-containing ether extract, neither the ether phase nor the water phase caused inhibition of fermentation. This indicates that the inhibitors are not alkali stable.

In (Parajò *et al.*, 1997-b) hydrolysates were vacuum concentrated to 25% initial volume for removing the fractions of organic solvents remaining in extracted hydrolysates.

As the main inhibitors have been localised to the non-volatile fraction a way to eliminate them would be to evaporate the stream to be recirculated that contains mostly water and volatiles. The non-volatile residue containing organic materials can be burned in a steam boiler to provide additional energy (Palmqvist *et al.*, 1996). Evaporation causes the removal of 10 or 90% of the initial volume. Addition of water is also required to restore the initial concentration of non-volatile compounds. pH adjustment to 5.5 with NaOH (10%w/v) was suggested (Larsson *et al.*, 1999-b). Steam stripping has been also suggested for removal of inhibitory compounds (Yu *et al.*, 1987).

Adsorption is a suitable strategy for improving the fermentability of lignocellulose hydrolysates (Frazer and Mc Caskey; Parajò *et al.*, 1996; Roberto *et al.*, 1991-a; Tran and Chambers, 1986). Parajò *et al.*, (1997-b) reported powdered charcoal was sequentially washed with 0.4 N HCl and water and dried at room temperature. Overlimed hydrolysates were mixed with 1.5 wt% of charcoal and stirred for 1 h at room temperature. Liquors were recovered by filtration and treated again for 1 h with the same amount of charcoal. The experimental results indicate that low adsorbent charges, such as in charcoal, significantly improve the fermentation when the

hydrolysate is already overlimed or treated with solvents. Charcoal adsorption is useful for removing part of the inhibitors remaining in the corresponding media.
several researchers tested the ion exchange detoxification procedure (Clark and Mackie 1999-b; Frazer and Mc Caskey, 1989; Fein *et al.,* 1984-b; Larsson *et al.,* 1999). Generally, the hydrolysate is treated with ion-exchange resins in a batch procedure. Larsson *et al.* (1999-b) treated the hydrolysate with polystyrenedivinylbenzene-based anion-exchange resin for 1 hour. The exchange resin was added until pH 5.5 or. The following ethanol yields were achieved after the anion-exchange treatment at pH 10 (0.49 g/g) and anion-exchange treatment at pH 5.5 (0.45 g/g). The productivity, however, was lower than the reference fermentation, which contained only glucose and nutrients (1.46 g/l /h). The pH in the anion exchange treatment determines different decrease in the concentration of fermentable sugars, 26 % at pH 10 and 8% at pH 5.5.
The detoxification procedure of ion exclusion was not frequently adopted. Some results are reported by Buchert *et al.* (1990).
Finally, some studies deal with detoxification for the use of several naturally or genetically engineered yeasts that can convert hexoses and pentoses to ethanol. It is well known that their capacity to ferment these sugars from acid hydrolysates of wood is hindered by the presence of inhibitors (Tran and Chambers, 1986; van Zyl *et al.,* 1988; Wang, 1994). The detoxification procedures previously discussed are usually employed. Sreenath and Jeffries (2000) removed acetate from acid hydrolysates of wood by using electrodialysis, according to the procedure reported by Datta (1989).

Chemical detoxification
Overliming has been the most widely used detoxification method (Leonard and Hajny; van Zyl *et al.,* 1988). Based on literature data, $Ca(OH)_2$ is generally selected as the agent for overliming (Roberto *et al.,* 1991-a; Roberto *et al.,* 1991-b). Calcium hydroxide is added to the *medium* and the hydrolysates are overlimed to pH 10-10.5. The precipitate consists mainly of calcium salts of low solubility dominated by calcium sulphate. The calcium sulphate sludge, containing precipitated inhibitors, is discharged and the detoxified hydrolysate is fed to the fermentation stage. The resulting precipitate is removed by filtration, and the pH set at 5.5 with sulphuric acid (Parajò *et al.,* 1997-b). The hydrolysate from steam-pretreated willow contains various substances that have an inhibitory effect on *E. coli* KO11. It is necessary to detoxify the hydrolysate prior to fermentation (Von Sivers *et al.,* 1994). The laboratory experiments show that the ethanol production rate was more than 10 times faster in an overliming-detoxified hydrolysate than in an undetoxified hydrolysate (Olsson *et al.,*).
The treatment can be combined with heat, because at elevated temperatures the solubility of calcium sulphate decreases and, in addition volatile compounds such as furfural are stripped off (Perego *et al.,* 1990). The effect of the combined treatment was probably due to decreased concentrations of Hibbert's ketones and aldehydes, and the removal of volatile compounds when a heat treatment was employed.
Detoxification by overliming of the non-volatile compounds improves the hydrolysate fermentability (Palmqvist *et al.,* 1996), as early detected by Leonard in his study on the fermentation of wood sugars to ethyl alcohol (Leonard and Hajny). A 36% increase of yield was observed. However, it was already pointed out that calculations have shown that the cost of overliming is 22% of the production cost of ethanol from pentoses in the analysis of ethanol production from willow using recombinant *Escherichia coli* (Von Sivers *et al.,* 1994).
Overliming has been proposed as a method for detoxification of hydrolysates owing to its ability to remove the same kind of compounds as neutralisation (Tran and Chambers, 1985), but with a marked beneficial effect derived from the precipitation of low molecular weight phenolics belonging to the acid-soluble lignin of wood. These types of compounds are inhibitorier than carbohydrate-degradation compounds (Leonard and Hajny, 1945). The detoxifying effect of overliming is due both to the precipitation of toxic components and to the instability of some inhibitors at high pH.

Treatment with a reducing agent such as sulphite, or a large yeast *inoculum*, are suggested as means to overcome unfavourable reduction potential in lignocellulosic hydrolysates (Leonard and Hajny, 1945). A combination of sulphite and overliming has been shown to be the most efficient method to detoxify hemicellulose hydrolysate (Olsson *et al.*, 1995). After treating with sodium sulphite (0.1% w/w) to obtain a *medium* with improved redox potential, the precipitate was removed by filtration and the pH was readjusted to 5.5 (Perego *et al.*, 1990).
Overliming under different conditions was compared by (Olsson *et al.*, 1996). According to method 1, the pH of the condensate is increased from 3.1 to 10.5 with $Ca(OH)_2$. Additionally, the condensate is supplemented with 1 g/l sodium sulphite. The mixture is kept at 90°C for 30 min. The solution is neutralised to pH 6.0 with concentrated sulphuric acid and centrifuged (4°C) for 10 min at 6500g. Method 2 adopts the same procedure as method 1 but the mixture is agitated for 1 h at room temperature, centrifuged for 10 min at 6500g, and, mixed with acid to give a final pH of 6.0. Method 3 is similar to method 2 with the exception that 3 g/l $Ca(OH)_2$ (75% of above) is used to increase the pH, this resultes in a maximum pH of 8.1. After overliming with method 1 and 2 no lactate formation was observed. After detoxification procedure with method 3, ethanol production rate was slowed down considerably by the inhibitors, and lactate production increased significantly after 32 hours. The reasons for the increased lactate production are not known.
Treatment with alkali is performed by adding 20% or 10 % (w/v) NaOH to pH 10. Then pH readjustment to 5.5 is made with H_2SO_4 and the hydrolysate is filtered (Larsson *et al.*, 1999-b). Adjustment of pH with NaOH decreases fermentability in comparison with adjustment with $Ca(OH)_2$. However, there is only little difference in the amounts of inhibitory compounds, but it is well known that monovalent ions such as Na^+ affect the ethanol productivity negatively.
In the treatment with sulphite the pH is at first adjusted with NaOH, after which Na_2SO_3, (0.1 or 1% w/v) at pH 5.5 or 10.1 (under an atmosphere of Helium) is added. The pH 10 is readjusted to 5.5 with H_2SO_4 (Larsson *et al.*, 1999-b; Olsson *et al.*,; Parajò *et al.*, 1996-a). The results show that to improve the fermentability a sulphite concentration higher than 0.1% is necessary. The addition of 0.1% sulphite is not sufficient to affect the amount of furan derivatives if calculated on molar basis, whereas the addition of 1% sulphite results in slight excess (Larsson *et al.*, 1999-b).
In detoxification by neutralisation, the pH adjustment to 6.5 is performed with $CaCO_3$. This procedure allows the maintenance of acetate and other acidic molecules in their unprotonated, less toxic form (Maiorella *et al., 1983;* Parajò *et al.*, 1997-b; van Zyl *et al.*, 1991). Moreover, neutralisation causes precipitation of toxic compounds found in hydrolysates, including heavy metals, furfural, acetate, tannins, terpenes and phenolics (Frazer and Mc Caskey, 1989; Perego *et al.*, 1990).
Sterile filtration was also tested for detoxification of hydrolysates. The condensate is extremely difficult to filter in normal laboratory filters. To facilitate the sterile filtration, the condensate is first centrifuged at 17000g for 1 h at 4°C and then prefiltered on 0.2 μm filters. Detoxification of the hydrolysate proved to be efficient in increasing the fermentability of the hydrolysate. However, it can be argued that the process economy will not allow for detoxification of the condensate.
The most important evidences of detoxification studies are outlined in these conclusions. Improved fermentability was observed for all detoxification methods. However, there were major differences between the efficiency of the detoxification methods as well as for the groups of inhibitory compounds removed by each method. In alkali treatment the adjustment of pH with $Ca(OH)_2$ increases fermentability more than adjustment with NaOH. There is only little difference in the amount of measured compounds. It is considered a confirmation that monovalent ions, such as Na^+, affect the ethanol productivity negatively, where Ca^{2+} does not. Neither acetic acid nor sugars were removed by treatment with alkali. The results also show that to improve the fermentability a sulfite concentration higher than 0.1% is necessary. The

enhancement in fermentability caused by addition of sulphite seems to be related with the adaptation of the unfavourable redox potential of hydrolysates (Parajò *et al.*, 1997-a).
Fermentability is increased after evaporation but the improvement is rather limited. Volatile compounds are not the major inhibitors; see also (Palmqvist *et al.*, 1996). Treatment with *T. reseei* improves the fermentability of a dilute-acid hydrolysate of spruce but, in contrast, the findings discussed by Palmqvist *et al.* (1997), proved that the treatment does not affect the concentration of acetic acid.
Treatment with laccase is specific for phenolic compounds. Ethanol yield is only slightly lower than that obtained after some other detoxification procedures, such as anion exchange. Ethanol productivity and biomass yield are still lower than in the reference fermentation. This could be owing to aliphatic acids, which are still present after detoxification. Furan derivatives are not removed by laccase, which results in an initial lag phase in ethanol production. Good fermentability can be generally achieved if phenolic compounds are removed from hydrolysate.
Treatment with anion-exchange resin is effective and gives good ethanol yield, productivity, and biomass yield owing to a significant decrease in concentrations of all the groups of inhibitors. This method, however, also removes the 26% of the fermentable sugars, most probably due to hydrophobic interactions with the matrix. A non-fermentable lignocellulosic hydrolysate, without treatment, could be fermented as quickly as a comparable glucose solution after ion-exchange treatment or charcoal treatment.
The toxins are mitigated sufficiently to allow fermentation by treatment with $Ca(OH)_2$ at 60°C but are not completely eliminated (Moniruzzaman and Ingram, 1998). The fermentability of Ca-treated hydrolysate is dependent on *inoculum* level and nutrients. Small amount of sugars remained late in the fermentation. The persistence of these sugars represents an opportunity for incremental improvements in the biocatalyst. Such improvements could potentially provide a small increase in product yield, a decrease in fermentation time, and a reduction in waste treatment.
Finally, the results outline another important conclusion. The process for the preparation of the hydrolysate is as important as the detoxification procedure.

Adaptation
An alternative approach to detoxification is to adapt the microorganism to the lignocellulosic hydrolysate. Adaptation has been shown to increase the ability of a broad range of yeast strains to grow in lignocellulosic hydrolysates (Fein *et al.*, 1984-b). Adaptation of *P. stipitis* CBS 5776 by repeated recycling in an acid hydrolysate from aspen resulted in an increase in ethanol productivity and yield. The same microorganism was also adapted to a detoxified hydrolysate and was then able to ferment an undetoxified hydrolysate (Tran and Chambers, 1986).
The ability to adapt *S. cerevisiae* to lignocellulosic hydrolysates has been shown to be strain dependent (Olsson and Hahn- Hägerdal, 1996). Isolation of strains from natural or industrial habitants has been shown to be a useful technique for finding strains with suitable properties for use in lignocellulosic hydrolysates. A strain of *S. cerevisiae* isolated from a sulphite-spent liquor (SSL) fermentation plant was shown to be able to utilize glucose and galactose simultaneously in the presence of acetic acid in contrast to the behaviour of Bakers' yeast (Linden *et al., 1992*). Of particular interest is the evaluation of high inocula fermentation (2×10^8 initial cells/mL) as a means of alleviating the inhibition problems (Chung and Lee, 1985). In continuous fermentation with cell recycle it is conceivable that cell death is counterbalanced by cell growth. Studies on rapid fermentation report that a phenomenal increase in productivity can be achieved using high cell density (Cysewski and Wilke, 1977; del Rosario *et al.*, 1979; Ghose and Tyagi, 1979). The toxicity problem of furfural could be overcome incontinuous fermentation with recycling of *S. cerevisiae* cells, a system that works with high cell density (9 g/l) (Navarro, 1994).
In addition, previous inhibition studies have confirmed that yeast cells take some of the toxins, especially furfural and HMF, up during ethanol fermentation (Azhar *et al.*, 1982; Morimoto and Murakami, 1967). The levels of furfural and HMF in the fermenter can decrease to only 3 and

10%, respectively, of those found in the feed at all dilution rate. This would provode a partial explanation for the low cell death in continuous fermentation.

The optimization of fermenter configuration and of fermentation conditions can favour the overcoming of toxicity and can be regarded as detoxification *in situ* (Taherzadeh *et al.*, 2000). At low feed rates, the specific rate of furfural is apparently high enough for the cells to be able to keep the furfural concentration low during the entire feed period. However, this is not quite the case for HMF. Fortunately, HMF has been reported not to be as toxic for the yeast as furfural (Sanchez and Bautista, 1988; Azhar *et al.*, 1981).

It was found (Taherzadeh *et al.*, 1999) possible to ferment severely inhibiting hydrolysates using a fed-batch technique. Furfural and HMF, and possibly other aldehydes, are converted to less inhibiting compounds. The compounds are mainly reduced to their corresponding alcohols, i.e., furfuryl alcohol and hydroxymethyl-furfuryl alcohol, which are less inhibitory than the aldehydes. The negative effects of these compounds can be largely avoided in fed-batch cultivations, provided that the feed rate matches the conversion rate.

The inhibiting effect of furfural is not only a function of the furfural concentration (Palmqvist *et al.*, 1999-a), but also of cell density (Navarro, 1994; Chung and Lee, 1985; Boyer *et al.*, 1992), culture conditions (fed-batch, batch or continuous fermentation) (Fireoved and Mutharasan, 1986; Villa, 1992), and aeration (Linden *et al.*, 1992). Adaptation *of S. cerevisiae* to furfural has been reported in batch (Banerjee *et al.*, 1981-a), fed-batch (Villa, 1992), and continuous culture (Chung and Lee, 1985). The adaptation might be due to the synthesis of new enzymes or co-enzymes for furfural reduction (Boyer *et al.*, 1992). Furfural is likely reduced to furfuryl alcohol-by-alcohol dehydrogenase (Weigert *et al.*, 1988).

The rate of furfural reduction has been reported to be proportional to the *inoculum* size (Boyer *et al.*, 1992) and to increase with increasing specific growth rate in continuous culture (Fireoved and Mutharasan, 1986). Most of these studies were conducted with industrial strains of normal bakers' yeast, because an industrial strain would be used for fermentation of furfural-rich substrate such as a lignocellulosic hydrolysate. When furfural is added to a chemostat culture, an adaptative response towards this compound is observed (Boyer *et al.*, 1992; Chung and Lee, 1985; Fireoved and Mutharasan, 1986; Villa, 1992). The experimental results and the mathematical modeling documented a twofold effect of furfural on the kinetics of glucose metabolism by *S. cerevisiae*. Furfural reduction to furfuryl alcohol is preferred to glycerol production as a redox sink. Therefore, furfural reduction causes more glucose to be available for ethanol production. In addition, by decreasing cell replication without a proportional effect on cell metabolism, furfural causes a larger proportion of glucose to be used for ethanol production. Furfural is metabolised by *S. cerevisiae* under aerobic (Taherzadeh *et al.*, 1998), oxygen-limited (Navarro, 1994) and anaerobic conditions (Palmqvist *et al.*, 1999-a). A mechanistic model describing the specific rates of growth, glucose consumption, ethanol and glycerol formation, and furfural reduction in batch fermentation has recently been developed (Palmqvist *et al.*, 1999-a), the model is based on the following assumption: 1) furfural reduction to furfuryl alcohol by NADH dependent dehydrogenases had a higher priority than reduction of dihydroxyacetone phosphate to glycerol, and 2) furfural caused inactivation of cell replication. The model accurately describes the experimental data in the absence and in the presence of furfural, strongly suggesting that the model assumptions are correct. The main conversion product of HMF metabolization is 5-hydroxymethyl furfuryl alcohol, suggesting similar mechanisms for HMF and furfural inhibition.

FERMENTATION

Fermentation is the term used to describe any process for the production of a chemical by means of the mass culture of a microorganism in the presence of proper nutrients (carbohydrates, lipids, purines and pyrimidines, vitamins and growth factors, amino acids, nitrogen and sulphur sources, chemical elements and inorganic ions).
The product can either be:

1. The cell itself: referred to as biomass production.
2. A microorganism's own metabolite: referred to as a product from a natural or genetically improved strain (*e.g.* Amino acids, Carbohydrates, Enzymes, Lipids, Nucleotides and precursors, Organic synthesis intermediates, *etc.*)
3. A microorganism's foreign product: referred to as a product from recombinant DNA technology or a genetically engineered strain, i.e. *recombinant strain* (*e.g.* Amino acids, Enzymes, Human therapeutics)

The scheme in Fig. 22 summarizes the general classification for microorganisms. The Protista kingdom comprises unicellular organisms both capable of duplication (Prokaryotes and eukaryotes) and of directing their own replication (non-cellular protists). Prokaryotes do not possess a true nucleus or a nuclear membrane, whereas eukaryotes have a nucleus enclosed within a defined nuclear membrane. The non-cellular protists are not able to self-replicate or control their reproduction within another cell termed the host. In particular, among the eukaryotes fungi may be subdivided into *lower fungi* as well as slime moulds and *higher fungi*, which include the *yeasts*. Yeasts are free-living single cells.
The criteria used for the classification of microorganisms include morphology, reproductive mechanisms, physiology, and means of culture. During the self-replication process, the microorganisms produce numerous complex macromolecules. Microorganisms that carry out their metabolism in the presence of oxygen are classified as aerobic. The anaerobic microorganisms are those able to metabolise the substances using nitrate, sulphate or ferric ions, instead of oxygen. Microorganisms can also be classified according to the lowest temperatures at which significant growth can be detected: *Psychrophiles* (T< 20°C), *Mesophiles* (20-45°C), *Thermophiles* (45-60°C). Many microorganisms display an optimum pH for growth at around 7, with a pH distribution in the 5-8 range.

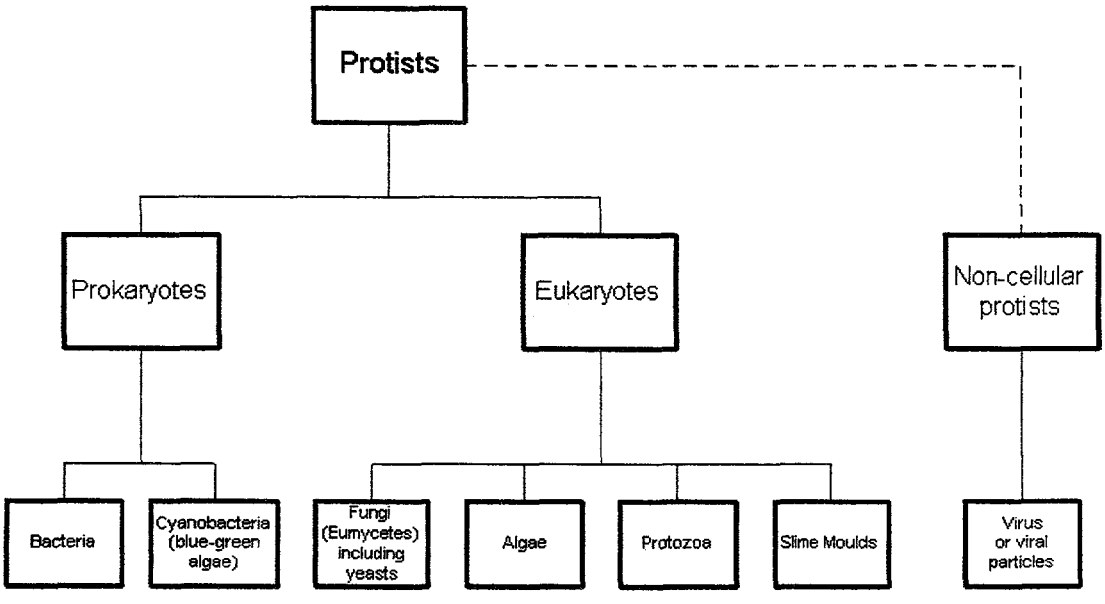


Fig. 22 - General classification of the microorganisms.

Fermentation systems can be divided into three categories: batch fermentation, fed-batch fermentation, and continuous fermentation.

Batch fermentation is a closed system in which the sterilized nutrient solution is inoculated with microorganisms at time t=0 and incubation is allowed to start. During the process the only additions are oxygen (in the case of aerobic microorganisms), antifoam agent, and acid or base to control the pH. The composition of the culture *medium*, and the metabolite concentration generally change constantly as a result of the cells metabolism.

Fed-batch fermentation adds the substrate progressively while the fermentation proceeds. In this process the nutrients are also added in small concentrations at the beginning of the fermentation and during the production phase.

Continuous fermentation is an open system. Sterile nutrient solution is added to the bioreactor continuously and an equivalent amount of the converted nutrient solution with microorganisms is simultaneously subtracted from the system.

The bioreactor where the mixing is carried out homogeneously is called a *chemostat* or *turbidistat* depending on which parameter has been chosen as the basis for the process. In the first, after reaching the steady state, adjusting the concentration of one substrate controls cell growth. In the second, cell growth is kept constant by monitoring the turbidity. This measurement allows for the continuous adjustment of the rate of feed in order to ensure a constant biomass concentration. Although the batch system for yeast growth and ethanol production can be used on an industrial scale, it suffers from a low volumetric efficiency. In this respect, the continuous process ensures the highest productivity and the fastest production rates. The limitation of this technology resides in the toxicity of the ethanol produced on the yeast activity. A simple procedure to rectify this is to remove the ethanol produced while it is forming. This can be achieved by carrying out the fermentation at low pressure in order to permit the instantaneous evaporation of the ethanol. Fermentation in a continuous reactor would lead in time to an inevitable dilution of the microrganisms responsible for the fermentation process. An effective way to control and maintain a high cell density is the use of *immobilized cell systems*. They immobilize the microorganism in some substrate that might be a solid support to which the cells are attached, a membrane or a polymeric gel embedded with the microrganisms.

SUGAR FERMENTATION TO ETHANOL

Glucose fermentation

Whatever the process employed to hydrolyse the biomass, the hydrolisate obtained is mainly constituted of glucose, xylose, arabinose, and cellobiose. The most widely employed microorganism in glucose fermentation is *Saccharomyces c.*, exceptional yeast since, in contrast to most other species it is able to perform alcoholic fermentation, and can grow under strictly anaerobic conditions. 'Non-*Saccharomyces*' yeasts usually require a growth-limiting supply of oxygen (i.e. oxygen-limited growth conditions) to trigger the alcoholic fermentation. Since it is very difficult to achieve an optimal oxygen level in large-scale fermentations, non-*Saccharomyces* yeasts are therefore less practical for large-scale alcohol production (van Dijken *et al.*, 1993).

The stoichiometric reaction for glucose conversion into ethanol is:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

Considering the fact that the molecular weight of ethanol is 46 g/mole and that of glucose is 180 g/mole and that one mole of glucose produce 2 moles of ethanol, the maximum yield is 2·46/180= 51% by weight. Scheme in Fig. 23 summarizes the metabolic pattern by which some microorganisms synthesize ethanol from glucose.

Although ethanol is the major product of *S. cerevisiae* when it is grown anaerobically, it can also exercise an inhibiting effect on the fermentation process itself. In general, ethanol concentration of around 10% seems to be sufficient to inhibit both growth and ethanol production in most yeast strains (Aiba *et al.*; 2000; Oliveira *et a.,l* 1998) to an extent, which depends on the nutritional status of the *medium*, as well as osmotic strength and temperature (Berry 1989). The identification of a microorganism that is also effective at relatively high ethanol concentrations is one of the goals to be reached in this field in order to improve the overall efficiency of the process.

Pentose fermentation

Cellulose biomass contains two major sugars, glucose and xylose. A major obstacle in this process is that *Saccharomyces* yeasts are unable to ferment xylose into ethanol. While technology to convert hexoses to ethanol is well established, the fermentation of pentoses is problematical.

In recent years, it has been sought (and identified) yeasts and fungi that can convert D-xylose into ethanol. However, the use of their cultures in the presence of pentose to obtain rapid and efficient ethanol production is somewhat more complex than the alcoholic fermentation of D-glucose with *Saccharomyces c.*. Among the factors strongly influencing ethanol yields in xylose fermentation, aeration is the most crucial. Tab. 23 lists some of the yeasts capable of fermenting xylose to ethanol with their respective performances.

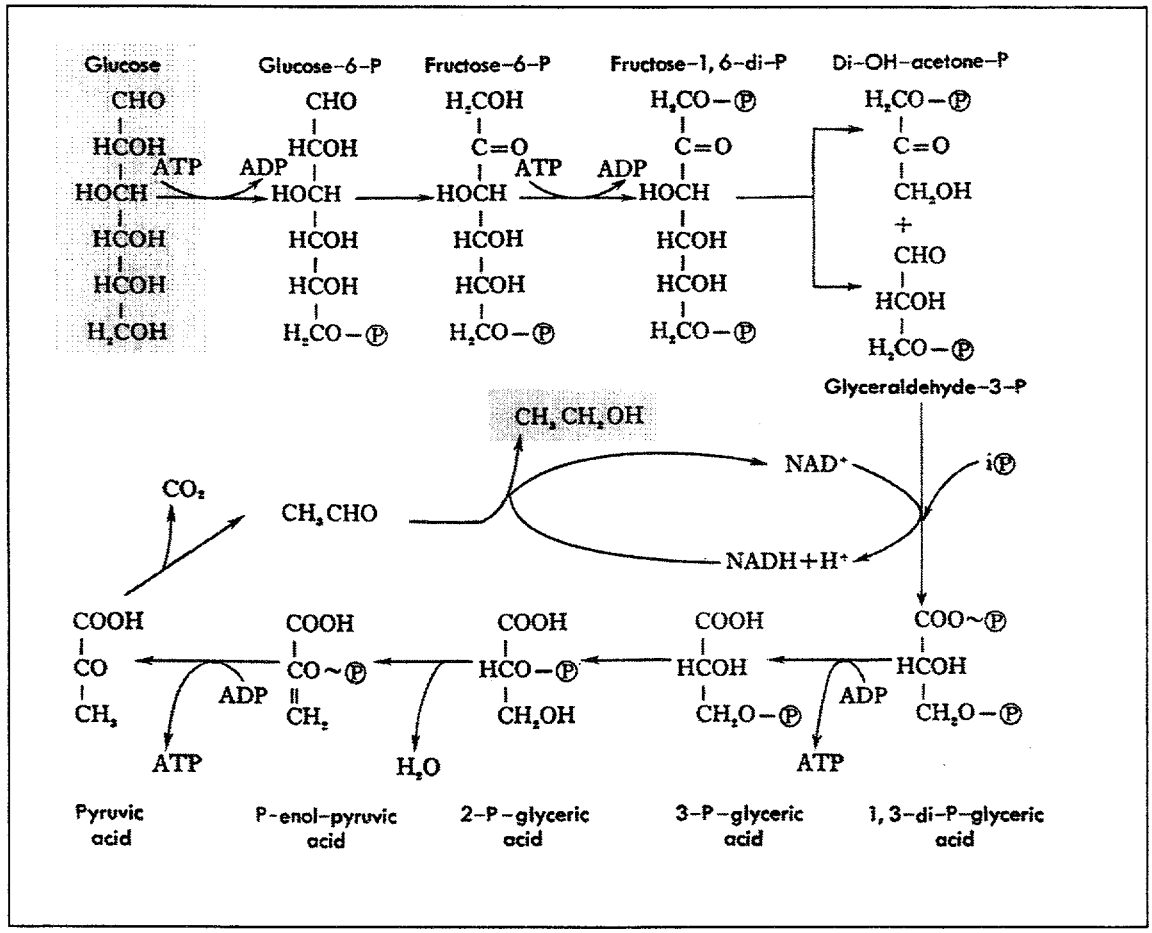


Fig. 23 - Metabolic pattern from glucose to ethanol in the *Saccharomices c.*

Tab. 23 - Yeasts capable of fermenting xylose to ethanol.

Yeast	**Xylose** g/l	**Yield**[a] g/g	**Productivity** g/l ·h	**Time** h	**Ethanol** g/l	**T** °C	*Reference*
Candida Shehatae NCL-3501	100	0.64	0.36	72	26.2	45	Abbi *et al.*, 1996
Pachysolen tannophilus NRRLY-2460 +CBS 4045	40	0.59	0.20	60	12.0	32	Converti *et al.*, 1998

[a]values computed with respect to the consumed sugars

Native microorganisms can convert xylose by means of two internal pathways schematically reproduced in Fig. 24. Both pathways provide a transformation of D-xylose to xylulose, which can in turn be converted to ethanol. The difference between them resides in the way by which microorganisms produce xylulose. One pathway provides a preliminary transformation to xylitol followed by a conversion into xylulose; the other ensures the direct conversion of xylose to xylulose. The reported process yields are not high (Chandrakant *et al* 1998) and have been improved by inducing some genetic modification into these microorganisms for the purpose of increasing the conversion activity of their enzymes.

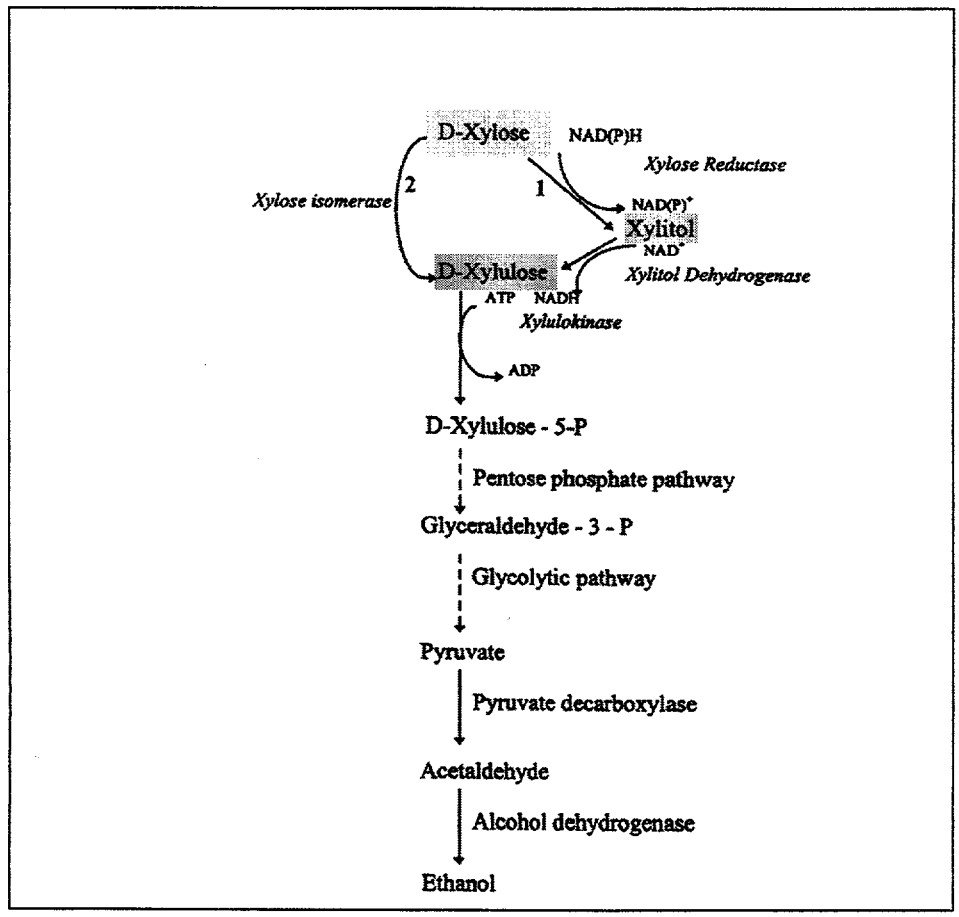


Fig. 24 - Metabolic pattern in the D-xylose fermenting microorganisms.

Three stoichiometric reactions have been reported with ethanol yields in the range of 30-50 % of the weight of the starter material.
The reactions usually considered for this conversion are as follows (note that II and III are not stoichiometric):

I. $3\ C_5H_{10}O_5 \rightarrow 5\ C_2H_5OH + 5CO_2$
II. $3\ C_5H_{10}O_5 \rightarrow 4\ C_2H_5OH$ + other prod.
III. $C_5H_{10}O_5 \rightarrow 2\ C_2H_5OH$ + other prod.

Usually the reaction stoichiometry considered in yield calculation is the first, it has a theoretical yield of 51% by weight, *i.e.* the same as computed for the corresponding glucose to ethanol fermentation.

Some thermophilic and mesophilic bacteria have been also identified which are able to ferment xylose to ethanol. Among these, the most thoroughly investigated are: *Clostridium thermohydrosulphuric and Clostridium ethanolicus* (Ogier *et al.*, 1999). They belong to the same bacterial family as the *Clostridium thermocellum*, which is unable to ferment xylose while showing very interesting properties for cellulase production. The use of these bacteria is not advantegeous for two reasons: the bacteria react poorly to certain levels of ethanol in the fermentation *medium*; the fermentation process is not selective and, in addition to the ethanol which is the most abundant constituent, some organic acids such as acetic acid are also produced. The reported yields for using *Clostridium ethanolicus* range from a minimum of 0.25g/g to a maximum of 0.50g/g, which means that the conversion yields are 49% and 98% respectively. Recently, another recombinant bacterium *Zymomonas mobylis* CP4 (pZB5) has been proven to be effective in co-fermenting both xylose and glucose (Krishnan *et al.*, 2000, Joachimsthal *et al.*, 2000, Lawford *et al.*, 2000). Krishnan *et al.* found that using a laboratory-scale fluidized-bed bioreactor (FBR) xylose conversion to ethanol was 91.5% for a feed containing 50 g/l of glucose and 13 g/l of xylose at a dilution rate of 0.24/h. NREL is currently devoting several research projects to the study of this bacterium.

Another viable process that can be used to ferment xylose to ethanol provides a preliminary conversion of xylose to xylulose and the subsequent fermentation of this to ethanol by means of common yeast like *Saccharomyces c.*. The first step is normally carried out by an isomerase enzyme, while the second by *S. cerevisiae*. The Tab. 24 lists the fermentation yields reported by Jeppsson and his team (1995).
The stoichiometry for the xylulose fermentation is reported below:

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2$$

whose fermentation yield is 0.51 (g/g)

Tab. 24 - Yeasts capable of fermenting the xylulose to ethanol in anaerobic conditions (Jeppsson *et al.*, 1995).

Yeast	*Xylose* g/l	*Yields* g/g	*Productivity* g/l·h	*Time* h	*Ethanol* g/l	*Temperature* °C
S. cerevisiae ATCC24860	50	0.68	0.12	72	8.75	30
p. stipitis CBS 6054	50	1.04	0.06	72	4	30
C. shehatae NJ 23	50	0.70	0.24	72	17.5	30

However, this process has the disadvantage of being limited by the difference between the optimal pH for the isomerase activity (pH 7) and the optimal pH for yeast activity (pH 5) apart from the cost of the isomerase enzyme.

Since 1980, numerous researchers have investigated the strategies of genetically modifying microorganisms capable of fermenting both xylose and glucose simultaneously. (Ho *et al.*, 1999). In addition to the *Zymomonas mobilis* previously cited, Ho *et al.*, developed strains of genetically engineered *Saccharomyces yeasts.* This was accomplished by redesigning the yeast's metabolic pathway for fermenting xylose to ethanol, including cloning three xylose-metabolizing genes, modifying the genetic systems controlling gene expression, *etc.* The engineered strains contain the following xylose-metabolizing genes (Ho *et al.*, 1998): a xylose reductase gene, a xylitol dehydrogenase gene (both from *Pichia stipitis*), and a xylulokinase gene (from *Saccharomyces c.*). As a result, these recombinant yeasts can not only effectively ferment both glucose and xylose to ethanol when these sugars are present separately in the *medium*, but can also effectively coferment both glucose and xylose present in the same *medium* to ethanol.
This result is an important milestone because the xylose uptake by microorganisms is repressed by the glucose, which is present in lignocellulosic hydrolysates apart from requiring low amounts of oxygen for optimal production (Jeffries *et al.*, 1999). Further experiments by Ho *et al.*, made it possible to create *super-stable genetically engineered* glucose-xylose-cofermenting *Saccharomyces* yeasts which contain multiple copies of the same three xylose-metabolizing genes stably integrated on the yeast chromosome. This is a development, which has made it possible for the genetically engineered yeasts to be effective for cofermenting glucose and xylose by continuous fermentation.

The performances of both yeasts and bacteria are affected by the composition of the fermentation *medium*, *i.e.* the activity of the microorganism is lower in a real hydrolysate than in a reference solution at the same sugar concentrations. This is due to the presence of inhibitiors in the hydrolysates responsible for a decrease in the microorganism activity and the expected yields. In this respect, the fermenting capacity of the microorganisms must be assessed as a function of the adopted hydrolysis route, pretreatment conditions and detoxification procedures. These comprise all the substrate treatments used to remove the inhibitors. The Tab. 25 summarises the xylose fermentation performances by xylose fermenting yeasts as a function of the hydrolytic process.

FERMENTATION STRATEGIES

Sequential fermentation of C5 and C6 sugars

One way of fermenting the hydrolysates is the sequential fermentation of first the C5 then the C6 sugars. Lindsay *et al.*, (1995) developed improved ethanologenic *Escherichia coli* strains for the fermentation of pentoses in sugar mixtures. Glucose-negative mutants of *E. coli* unable to ferment glucose were isolated. By this approach, a mixture of hexose and pentose sugars was fermented with near theoretical yield by the SL40 strain.

Separate hydrolysis and fermentation

Another strategy often employed is the Separate Hydrolysis and Fermentation process (SHF). This is a typical sequential steps process in which the cellulose is first hydrolyzed into C6 sugars, which are in turn fermented into ethanol. This process shows the intrinsic advantage of allowing the enzyme and the microorganisms to work in optimal pH and temperature conditions. However, the inhibitory effect of glucose on microorganism activity and the disadvantage of operating with two bioreactors should be considered.

Simultaneous Saccharification and Fermentation

One way to solve the problem of inhibition by glucose is to carry out the hydrolysis and fermentation simultaneously. The overall process is referred to as SSF (Simultaneous Saccharification and Fermentation). The primary advantage is the increase in the hydrolysis rate due to decreased product inhibition, as cellulase is inhibited by glucose and cellobiose. Beside, the lower cost resulting from the reduction in the number of reactor vessels needed means this process is cheaper than SHF. The major technological issue to be solved lies in finding the proper combination between the optimal operational condition and pH and temperature values for both the enzyme and the microorganism.

The key condition is the temperature. In fact, while the cellulase enzymes are more active at 50 °C, the yeast will usually only work at a temperature no higher than 35°C. Research has concentrated on the isolation of strains able to work at higher temperatures. Promising performances have been recently discovered in certain strains of *S. cerevisiae* that can operate at 37 °C (Bollok *et al.* 2000) and in some strains of *Kluyveromyces marxianus* yeast (Ogier *et al.*, 1999). Abdel-Fattah and his team (2000) selected several strains capable of growth at 40-43°C. The two best-performing strains, a *Saccharomyces c. F111* and a *Kluyveromyces marxianus WR12* were used for industrial ethanol production in an Egyptian distillery using sugar cane molasses. Mean ethanol production was 7.7% and 7.4% (w/v).

Fermentation in co-cultures

Both the SHF and SFF process described above are concerned solely with the fermentation of the C6 sugars, whereas a complete conversion should also take into account the fermentation of C5 sugars. If the hydrolysis of cellulose and hemicellulose has been conducted separately, then the fermentation of the two hydrolysates obtained can also be carried out separately or sequentially. The optimal condition would be the simultaneous fermentation of both sugars. As already discussed, the fermentation of xylose can be carried out by *Pichia stipitis* and *Candida shehatae* thanks to their relatively high conversion efficiency. However the same microorganisms display very low activity in the fermentation of glucose. The problem can be solved by using a *medium* containing the microorganism for the fermentation of both xylose and glucose (*co-cultures*).

The major research projects on this topic have been focused on the yeasts *S. cerevisiae* and *P. stipitis* (Ogier *et al.*, 1999). However, several problems have still to be overcome before this strategy can be economically applied. In fact, when xylose and glucose are present in the same reaction *medium*, the glucose fermentation occurs at an initial ethanol producing level that can inhibit the activity of the yeast specific for xylose. Moreover the glucose fermentation uses some of the oxygen, which is crucial in the fermentation of xylose. Finally, we have to remember that incompatibility between two different microorganisms can dramatically reduce the activity of one of them.

Simultaneous Saccharification and Cofermentation

Some promising results have been obtained by Lebeau *et al.*, (1998) by using a continuous alcoholic fermentation (*Simultaneous Saccharification and Cofermentation, SSC)* in a co-immobilized system configuration containing cultures of *S. cerevisiae* and *C. shehatae*. The fermentation of a mixture consisting of 35 g/l glucose and 15 g/l xylose gives 100% conversion of glucose and 73% of xylose with an ethanol yield from the total sugar concentration of 0.48 g/g. However, the productivity is low (0.37 g/h·l) and should be improved.

Tab. 25 - Xylose fermentation performances of yeast in real hydrolysates.

Yeast	Biomass	Hydrolysis	Sugars g/l	Overall Yield[a] g/g	Productivity g/l ·h	Time h	Ethanol g/l	Ref.
Pachysolen tannophilus (NCL-3501)	Rice Straw	dilute acid (overall)-free cell	20	0.37	0.15	48	7.2	Abbi *et* al. 1995
Pachysolen tannophilus (NCL-3501)	Rice Straw	dilute acid (overall)-immob.ed cell	20	0.47	0.22	48	10.6	Abbi *et* al. 1995
Pachysolen tannophilus ATCC 32691	Barley Straw	ammonia fiber explosion+enzym.hydr. (overall)	20(51% glu + 26%xyl)	0.95	0.13	45	6.3	Belkacemi *et al.*, 1998, Savoie *et al.*, 1998
Pachysolen tannophilus ATCC 32691	Corn Stalks	ammonia fiber explosion+enzym.hydr. (overall)	20(47% glu + 22%xyl)	0.75	0.08	45	3.7	Belkacemi *et al.*, 1998, Savoie *et al.*, 1998
Pachysolen tannophilus ATCC 32691	Alfalfa	ammonia fiber explosion+enzym.hydr. (overall)	20(41% glu + 34%xyl)	0.10	0.12	45	5.7	Belkacemi *et al.*, 1998, Savoie *et al.*, 1998
Pachysolen tannophilus ATCC 32691	Timothy	ammonia fiber explosion+enzym.hydr. (overall)	21(45% glu + 30%xyl)	0.53	0.08	45	4.4	Belkacemi *et al.*, 1998, Savoie *et al.*, 1998
Pachysolen tannophilus ATCC 32691	Reed Canary grass	ammonia fiber explosion+enzym.hydr. (overall)	20(48% glu + 28%xyl)	0.87	0.11	45	5.2	Belkacemi *et al.*, 1998, Savoie *et al.*, 1998
Pachysolen tannophilus DSM 70352	Wheat Straw	Chemical delignification by NaOH (overall)	27 (10.38 xyl +16.62 glu)	0.44	0.25	47	11.8	Zayed *et* al 1996
Pachysolen tannophilus NRRLY-2460 +CBS 4045	Oak	hemicellulose acid-hydrolysates	52.5 (43,5 xyl +9 glu)	0.51	0.07	168	11.2	Converti *et* al 1998
C. shehatae (CSIR-22984)	Red Oak	hemicellulose acid-hydrolysates	43 (40 xyl+3 glu)	0.25	0.05	200	10	Ogier *et* al 1999
P.stipitis (NRLL-Y7124)	Eucaliptus	Dil. Acid hydrol	32 (30.5 xyl + 1.5 glu)	0.35	0.16			Ogier *et* al 1999
Candida shehatae ATCC 22484	Hardwood	hemicellulose acid-hydrolysates	62 (43.5 xyl+9.0glu)	0.75	0.17	96	16	Perego *et al.*, 1990
Pachysolenum tannophilus (NRLL Y 2460)	Hardwood	hemicellulose acid-hydrolysates	61.6 (43.5 xyl+9.0glu)	0.59	0.15	120	18	Perego *et al.*, 1990

[a]values computed with respect to the utilized sugars.

KEY ECONOMIC ASPECTS OF PRODUCING ETHANOL FROM LIGNOCELLULOSIC BIOMASS

Pretreatment cost

The step of the lignocellulosic pre-treatment (*e.g.* Steam Explosion) is much more expensive than that used in the production of ethanol from sugar and starch (slicing, milling, *etc.*).

As regards the process used to get a substrate suitable for the enzymatic hydrolysis an analysis of the costs associated to the straw processing is reported by Fan *et al.* (1982) and summarized in Tab. 26

Tab. 26 - Cost analysis of some pre-treatment methods (Fan *et al.*, 1982).

	Yield of sugar g/kg_{straw}	**Pretreatment Cost** $\$/kg_{straw}$
Caustic	341.0	0.04
Peracetic acid	279.9	7.51
Sulphuric acid	140.5	0.11
Ball-milling, 8h	255.98	0.01
γ–irradiation 50 Mrads	207.02	0.1

Enzyme cost

Looking at the overall process for ethanol production, the cost of cellulose enzyme hydrolysis continues to be the major economic barrier especially in view of the fact that the enzyme activity may be significantly lowered by the presence of inhibiting molecules generated by the pre-treatment adopted.

One way to significantly reduce enzyme production costs is the on-site synthesis and utilization. Here Cellulase is most likely to be produced separately, using the hydrolyzate from pentose sugars resulting from the pretreatment and/or glucose from the cellulose hydrolysis. Fig. 25 represents a schematic flow diagram of this process (Philippidis *et al.*, 1997).

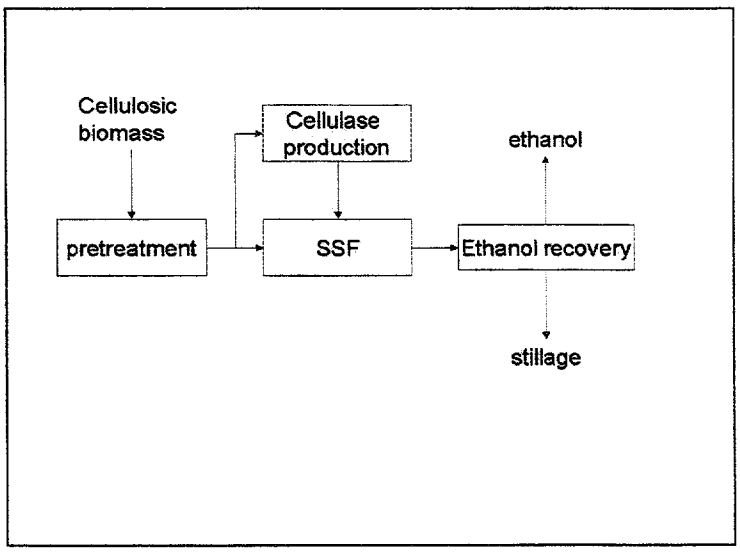


Fig. 25 - Schematic flow diagram for the on site enzyme production and utilization.

Some researchers have tried to produce the enzymes on-site starting from microorganisms in a *medium* containing sludge of the pretreated biomass. Tests were carried out on paper sludge, pretreated wood (steam exploded), and their hydrolysis residues, in shake flasks and fermenters to assess their respective productivity and titers (Shin *et al.,* 2000). Among the substrates explored, steam-exploded wood performed the best as a substrate in cellulase production with 2% (w/v) slurry in the shake flask.

Another way of reducing the amount of enzyme required is *to recover and reuse* the enzymes required for the hydrolytic step (Lee, D., *et al.,* 1994). The first step in cellulose hydrolysis is adsorption onto the cellulosic substrate. As hydrolysis proceeds, a portion of the adsorbed enzymes is gradually released into the supernatant. Therefore, cellulase can be recovered from either phase of the reaction. The simplest method for cellulase recovery is reabsorption of the enzyme present in the supernatant onto a fresh substrate. Typically a temperature of 4°C and a contact time of 60 min. is enough to reach adsorption equilibrium. In order to recover enzymes from the substrate many strategies have been explored. The most effective provide for alkali extraction using Tween-80 or just put the old substrate in contact with a fresh one. The recycle is then repeated after 12 h hydrolysis. The extent of enzyme recovery is strongly influenced by the lignin content in the treated substrate.

An economically viable process should provide *almost total hydrolysis of the cellulose and hemicellulose* components. It should be mentioned that the hemicellulose streams contain different levels of sugars depending on the type of biomass and, besides xylose, contains arabinose, galactose, glucose and mannose. The enzymes responsible for the hydrolysis of xylan are xylanase (endo 1,4-β-D-xylanohydrolase, EC 3.2.1.8) and β-xylosidase (endo 1,4-β-D-xylohydrolase, EC 3.2.1.37) (Saraswat *et al.,* 1997). Xylanases cleave the β-1,4 linkages on the xylan backbone in an endo manner, β-xylosidase hydrolyses xylobiose and small xylooligosaccharides to xylose and facilitates the hydrolysis of xylan by removing the inhibition of the xylanase. A few additional debranching enzymes are also required to complete the hydrolysis of xylans by removing of the side chain substituents from the xylan backbone. At least seven enzymes are required for complete hydrolysis. Besides the three previously mentioned for cellulose (*endo-* and *exo*-gluconase, and β-glucosidase), four other enzymes are required to debranch hemicellulose. Xylanase can be produced from a big number of strains (Medeiros *et al.,* 2000). In particular, *Trichoderma harzianum* strains T4 and T6, *Acrophialophora nainiana*, and *Humicola grisea* var. *thermoidea* were selected for their ability to produce carbohydrate-degrading enzyme activities in a *medium* containing banana plant residue as the carbon source. Thermophilic fungi producing extremely high β-xylanase levels and their associated hemicellulases have attracted considerable attention because of potential industrial applications (Singh *et al.,* 2000). *Thermomyces lanuginosus strain SSBP* isolated from soil, produced β-xylanase when cultivated on a *medium* containing corncobs as the substrate and yeast extract as the nitrogen source. This xylanase is stable at up to 70 °C and at pH 5.5 ÷9.0 for 30 min whereas the other hemicellulases are less stable. These results suggest that the most suitable conditions for the hydrolysis of hemicellulose by these enzymes would be at 50 °C and pH 6.0. Another fungus isolated from soil is *Aspergillus flavus* (de Souza *et al.,* 1999). The enzymes worked best at pH 5.5÷ 6.0 and were highly active and stable under alkaline conditions. Among the microorganisms capable of producing both cellulase and xylanase enzymes, *Aspergillus niger KKS* was found promising in several bioreactor configurations (Kim *et al.,* 1997). Tab. 27 lists the maximum and minimum enzyme yields obtained by Kim and co. using a substrate of rice straw and different types of bioreactor configurations.
Several biotechnology companies are working in the field of enzyme preparation for ethanol production from biomass. Genencor International (USA) is involved in the development of low-cost cellulases and other related enzymes for the conversion of biomass into ethanol, and has recently been awarded a $17 million grant by the National Renewable Energy Laboratory for the

next few years. Novo Nordisk Biotech, located in Davis (California) is a wholly owned research and development subsidiary of Novo Nordisk A/S (Denmark) the world's largest producer of industrial enzymes, among its commercial products, Cellubrix® is a cellulase and cellobiase preparation for transferring cellulose material into glucose.

Tab. 27 - Maximum and minimum enzyme yields from *Aspergillus niger* KKS (Kim *et al.*, 1997)

	Yield ($IU/g_{protein}$)		**Productivity** (IU/l·h)	
	Min	*Max*	*Min*	*Max*
Cellulase	34	84	3.1	9.7
β-glucosidase	124	370	16.7	26
xylanases	444	9100	417	823

Iogen a private sector biotechnology company which is profitably marketing industrial enzymes in the pulp and paper, textiles and animal feed industries, has built a $25 million ethanol-from-cellulose *demonstration* plant adjacent to its enzyme manufacturing plant in Ottawa (Canada), its partners include Petro-Canada and the Government of Canada. Finally, Fermetech (Russia) is currently conducting research into enzyme production.
This enzyme market is yet to be developed and represents a significant opportunity only if the economics of lignocellulosic hydrolysis become more favourable.
In this area, several issues must be addressed. Among them, the most important are market size, profit margins, synergy with business, resources required, benefits, strengths, weaknesses, opportunities, threats *etc.*
The World enzyme market is estimated to be $1.4 Billion and constituted as shown in Tab.28 (Hettenhaus *et al,* 1997),

Tab. 28 - The composition of the world enzyme market (Hettenhaus *et al.*, 1997)

Field of application	**Million dollars**
Detergents	462
Starch	163
Textiles	150
Other	584
Total	**1400**

(Source: Novo Nordisk*)*

Nowadays, the infrastructure required to support the fuel ethanol industry is still incomplete. One major deficiency is represented by the poorly planned raw material supply as regards both composition and quantities. In fact, the enzymatic conversion of cellulose to get ethanol is more difficult than the starch-based procedure. In particular, cellulose conversion requires almost 30 times more enzymes for an equivalent amount of starch. For this reason, a larger biomass market is needed to give a strong boost to enzyme industry development. Besides this problem, enzyme purchasing also is a crucial factor for industrial development. In particular, very important building blocks for the development of the industrial bio-ethanol process are enzyme

performances in the process, the conditions for best process performance, the cost of hydrolysis, $/gallon of Ethanol and Strain or Process Licensing *etc.*
There are limited opportunities for lowering the cost of producing cellulase. Hettenhaus *et al.*, (1997), have estimated a cost of $5/kg enzymatic protein for the protein contained in the fermentation broth. The production cost does not include any downstream processing costs such as filtration, concentration, blending and shipping (Tab. 29)

Tab. 29 – Enzyme fermentation costs, **$/liter** (Hettenhaus *et al.*, 1997)

Fermentor Size	$150m^3$	$300m^3$	$500m^3$
Raw Material	0.14	0.14	0.14
Direct Cost	0.12	0.08	0.07
Equipment Depreciation	0.06	0.05	0.04
Total Fermentation Cost	**0.32**	**0.27**	**0.25**

As can be inferred from the reported figures, increasing the size of the fermentor provides economies of scale. However, the high viscosity of the broth requires a robust agitation system. The viscosity of the broth becomes a denaturation source for the enzymatic proteins with increased reactor size
Increasing the size to 500 m^3 reduces the cost to $0.25/liter or $3.90/kg. However, in increasing the size of the fermentor, an on-site enzyme production should be considered because road transportation of big tanks containing the product becomes prohibitively expensive. In fact, if produced and used on site, the enzyme liquor only has to be conveyed to the hydrolysis tanks. In addition, if produced elsewhere, it needs storage. This procedure requires the addition to the liquor of compounds to prevent microbial contamination and protein denaturation. The most commonly used for this purpose are sodium benzoate and glycerol, respectively, however, the use of these chemicals to stabilise the enzyme liquor inevitably leads to an increase in the production costs.
In Tab. 29 *lactose* was assumed as the *raw material*. Its market cost is twice that of glucose, and it is in limited supply. The use of the hydrolyzates from the process would be much cheaper and should reduce the cost to almost $3/kg. Among the companies already involved in the production of enzymes using glucose as a substrate instead of galactose, Iogen is the leader (relevant data has still to come from Iogen).
Recent analyses from CEA (Chief Executive Assistance) devoted to the marketing of emerging applications of biotechnology indicate the parameters that can be improved to reduce production costs. Tab. 30 lists the parameters to be examined with their respective improvements in costs. The main factors responsible for enzyme cost are: fermentor size, cost of the raw material on which they are grown, the protein expression[2], productivity and fill capacity[3].

[2] The protein expression is the amount in g/l of proteins, which can be separated from the fermentation broth by means of compression under conditions that permit the liquid to escape while the solid (the proteins) is retained. Usually this value ranges from 60-70 g/l.
[3] The fill capacity is the maximum percentage of the enzyme proteins that can be reached in a fermentation broth without exceeding viscosity limits. Usually this is around 80%

Tab. 30 - The main parameters to be optimised in order to lower enzyme production costs.

Improvement factors	Benefit, $/kg	Difficulty	Effort
Larger Fermentors, 300 m^3 to 500 m^3	$0.80 to $1.10	Low	low
Glucose or Hydrolyzate Substrate	$1.00 to $2.00	Low	Low
Protein Expression, 80 g/liter	~$1.00	High	High
Productivity, Fill Capacity, Cycle time	< $1.00	High	High

(Source: CEA)

Besides the process factors, other improvements can be achieved by acting directly on the enzyme itself in order to optimise its activity. There are many ways to increase the specific activity of the enzyme. One can:

- Increase the Thermal Stability of the enzyme.
- Decrease Non Specific Binding.
- Decrease Feedback Inhibition.
- Enhance Enzymatic Decrystallization, *e.g.* higher pH, more effective cellulase binding domain.

Hettenhaus *et al.,* (2000) reported that in order to make lignocellulosic conversion to ethanol more economically viable, the enzyme cost should be lowered from the 50 ¢ per gallon of ethanol identified in 1997 to no more than 5¢. Moreover, even if research has significantly advanced in the field of xylose fermentation, none of the available strains have been used on a demonstration scale. Iogen has targeted 80 % xylose (Hettenhaus *et* al. 2000).

Feedstock and plant cost

Some of the approaches for biomass conversion to ethanol examined seem very nearly ready for the commercial use but none of them have yet been demonstrated. As already outlined, the most relevant worldwide technologies are the concentrated acid produced by Arkenol and Masada, the dilute acid produced by BC International and Iogen's acid-enzyme hydrolysis. The acid technology is already mature and needs very few improvements in the process economics. By contrast, the enzymatic process needs further improvement if enzyme production costs are to be lowered.

Recently Kaylen and his team (2000) reported a detailed economic assessment of the conversion of lignocellulosics to ethanol and furfural using the dilute acid technology. The authors reported that the process starts to be economically viable only if the production of furfural from hemicellulose is taken into account. The Tab. 31 lists the biomass types considered.

Tab. 31 - Biomass feedstock for ethanol production by means of dilute acid hydrolysis technology (Kaylen *et al,.* 2000).

Feedstock	**Unit price** ($/ton)
Corn stalks	25
Grain sorghum stalks	25
Energy crops (5 ton/acre)	44
Wheat straw	25
Primary wood processing (hardwood)	25
Logging residues	25

Data reported in the Tab. 31 do not include the costs of the transportation, which have been calculated according to the following expression: *$0.15·ton·miles*. The base case prices used in the model are: ethanol $ 1.25 per gallon, and furfural $ 640 per ton.
Tab. 32 summarise the estimated costs.

Tab. 32 - Costs estimation for the production of ethanol from lignocellulosics (Kaylen *et al.*, 2000).

Capital investment	$455 million
Plant size	4360 tons
Operating costs	$92.2 million
Feedstock	$36 million
Transportation	$44.8 million
Produced ethanol	47.5 million gallons
Produced furfural	323 thousand tons
Ethanol price	1.25 $/gallon
Furfural	640 $/ton
Annual income	$281 million
Operating profits[a]	$108 million
Internal rate of return	22.6%

a) On an annual pre-tax base.

The internal rate of return 22.6 % indicates that the plant is worthy of consideration.

This analysis has focused exclusively on the technological aspects of the conversion of lignocellulosics to ethanol. However, in order to get a more complete picture, government policies have also to be considered. At present the bioethanol market is strongly dependent on clean air issues and state subsidies (for example in the US) are apparently destined to expire in 2007 and no further program is planned.
A comparative study of the projected costs associated with the several technologies for ethanol production was carried out by the Department of Business, Economic Development & Tourism in the State of Hawaii. Some of the obtained results relevant to a bioethanol production of 25 Mlgal/y are plotted in Fig. 26.

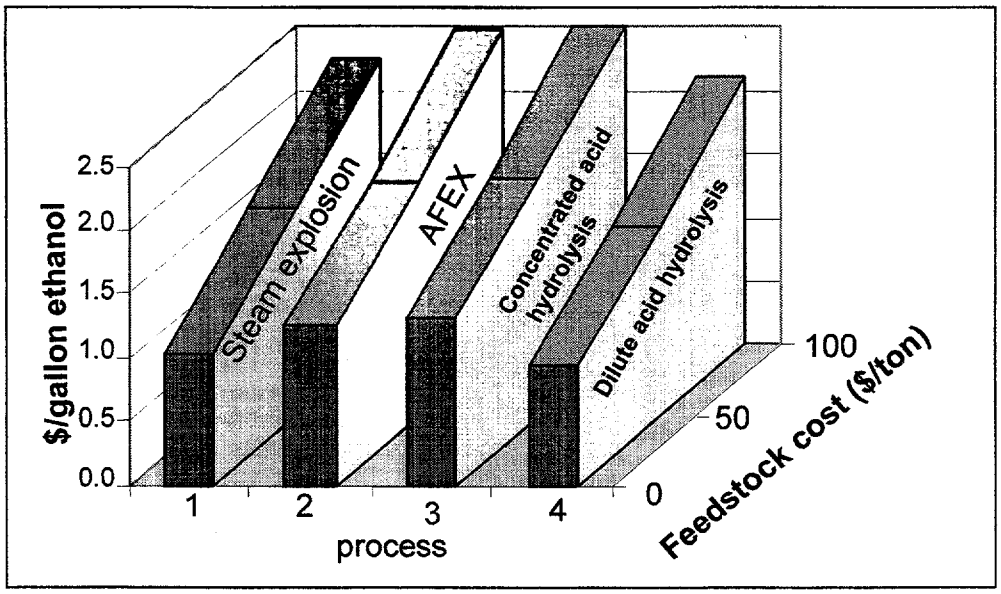


Fig. 26 - Bioethanol projected costs for a 25 Ml gal/y plant (*Report from State of Hawaii, Department of Business, Economic Development & Tourism, 1994*)

Data reported suggested that the projected ethanol costs range between 0.94 $/gal to 2.5 $/gal. Only slight variations can be observed belonging to the employed technology while a more significant influence is due to the feedstock cost. However, in comparing the projected costs for the several technologies, it should be kept in mind that whereas process 3 and 4 (those using the chemical hydrolysis) are well-defined technologies, process 1 and 2 (those using the enzymatic hydrolysis) are still focus of technical research. Then, low ethanol costs are expected from the technical advances in the enzymatic hydrolysis.

Some recent confidential elaboration from Bryan & Bryan Inc. report that the projected ethanol price for the dry milling technology ranges between 1.236 $/gal and 1.470 $/gal depending on the corn, the natural gas, and the electricity price other than the contribution of the taxes and insurance. Data obtained are relevant to a 15 millions gal per year plant size. Similar evaluations can be carried out for a 15 millions gal plant producing ethanol from lignocellulosic by steam technology followed by hydrolysis and fermentation using both data from Staketech company and the Hawaii State report. The analysis of the reported figures leads to ethanol production costs ranging between 1.14 $/gal and 1.68 $/gal depending on the biomass costs (calculations have been carried out for 0 $/ton and 50 $/ton respectively). These evaluations suggest that the projected cost associated with the two compared technologies (the one from corn and the other from lignocellulosics) overlays somehow depending on the boundaries conditions.

Fig. 27 compares the influence of the different expenses for the production of ethanol from a lignocellulosic feedstock and a corn feedstock. The results shows that while the major obstacle to the commercialization of ethanol from corn is the feedstock price, one of the major expenses affecting the ethanol production cost from lignocellulosics is the enzyme cost production. However, while the cost of enzyme technology could be lowered thanks the technical advances, that of corn could be 0.655 $/gal in the best case.

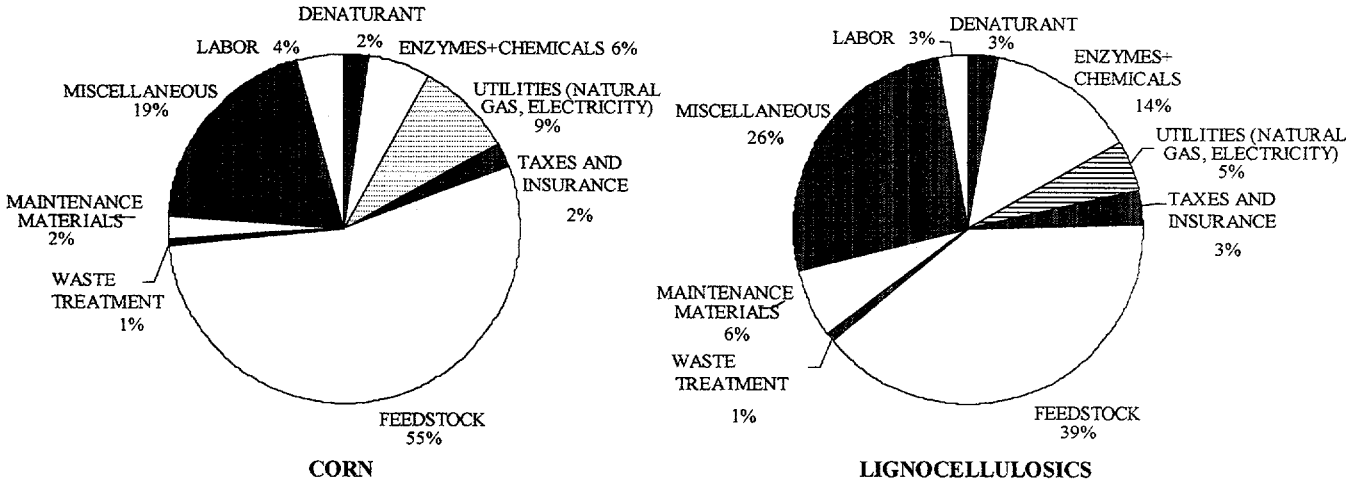


Fig. 27 – Percent contribution of the several expenses to the ethanol production costs in the case of starch feedstock (corn) and lignocellulosics.
(Data for corn are from Bryan & Bryan Inc. Data for lignocellulosic are from Wyman et al., 1993)

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