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Ethanol and glucose
tolerance of *M.indicus* in
aerobic and anaerobic conditions

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Abstract

Over the last few decades, ethanol production from renewable resources has been of interest as an alternative fuel to the current fossil fuel, due to the unstable oil market and in order to decrease net emission of carbon dioxide which leads to global warming. According to analyses of DG Transport and Energy (TREN), it is not possible to reach the current biofuels directive promoting 5,75 % biofuel by the year 2010, due to the markets and technologies, but by the year 2020 achievement of 6.9% is expected. This new law will increase biofuel demand by 3,1 %.

Lignocelluloses materials, which are relatively cheap and plentiful, are considered to be the main source of feedstock's for low-cost bio-ethanol production. The general procedure to convert lignocelluloses material to bioethanol is hydrolysis of the hemicelluloses and the cellulose to its monomer sugars, fermentation and distillation. Bacteria, yeasts and filamentous fungi are able to ferment hydrolysates from different plants and convert it to bioethanol.

Mucor indicus is a filamentous fungus; it is able to utilize a wide range of hexoses, pentoses and disaccharides (cellobiose) in order to produce ethanol. The Ethanol yield and productivity of this microorganism from hexoses are as same as *Saccharomyces cerevisiae*. But the reason that it is one of the candidates for ethanol production is the fungus ability to utilize xylose. The cell wall of *M.indicus* contains significant quantity of chitosan/chitin which can be easily extracted. Chitosan is the deacetylated products of chitin. They have many applications in chemistry, biotechnology, medicine, veterinary, dentistry, agriculture, food processing, environmental protection, water purification, cosmetic and textile industries.

The results of the current work show that the glucose concentration in the medium had a great impact on the lag phase, glucose consumption and ethanol production in both aerobic and anaerobic conditions. The lag phase increased as the initial concentration of glucose increased. While the glucose concentration increased above 190 g/l in the medium the glucose consumption and ethanol production decreased in both aerobic and anaerobic conditions. The glucose tolerance of *M.indicus* in both aerobic and anaerobic condition is about 190 g/l and in the anaerobic condition the ethanol tolerance of this fungus is around 70 g/.

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1. Introduction

1.1. Biofuels and the environment

Today, most cars and trucks on the road use gasoline and diesel as their fuels. It is believed that fossil fuels are limited; it is not replenished at the rate that it is consumed. Combustion of petroleum-based fuels increases net emission of carbon dioxide, different toxic and volatile compounds that are responsible for the health hazards and pollutions such as; benzene, toluene and xylenes[1].

One of solutions to that is to use biofuels made of renewable materials such as lignocellulosic materials in transportation instead of fossil fuels. Bio-ethanol can be used to replace all or part of the fuel for engines designed to gasoline. It has a higher octane rate than gasoline, which improves fuel combustion and decrease emissions of CO, NOX and hydrocarbons [1, 58]. It decreases net emission of carbon dioxide which leads to global warming and also reduces the dependence on non-renewable oil. The carbon dioxide is taken up by growing plants in the process of photosynthesis and stores in the form of starch, the plants-biomass- can be converted to biofuel and be used for transportation (figure 1).

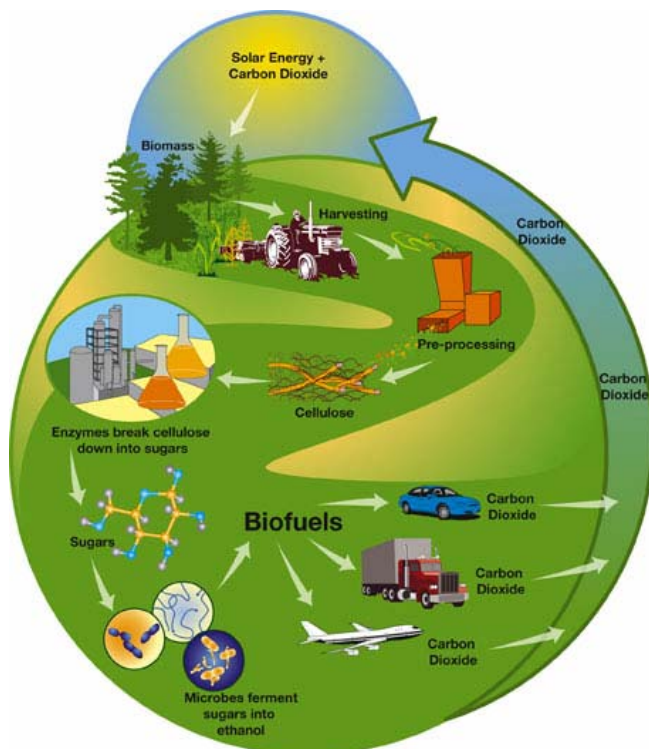


Figure 1: carbon cycles of bio-ethanol [2].
Adapted from [48]

1.2. Bioethanol in the world

Brazil and USA are the biggest producers of bioethanol in the world. In the EU, Spain, France and Sweden are the major ethanol producers and in Asia is China and India.

According to analyses of DG Transport and Energy (TREN), it is not possible to reach the current biofuels directive promoting 5,75 % biofuel by the year 2010, due to the markets and technologies, but by the year 2020 achievement of 6,9% is expected. This new law will increase biofuel demand by 3,1 %. [3], (figure 2).

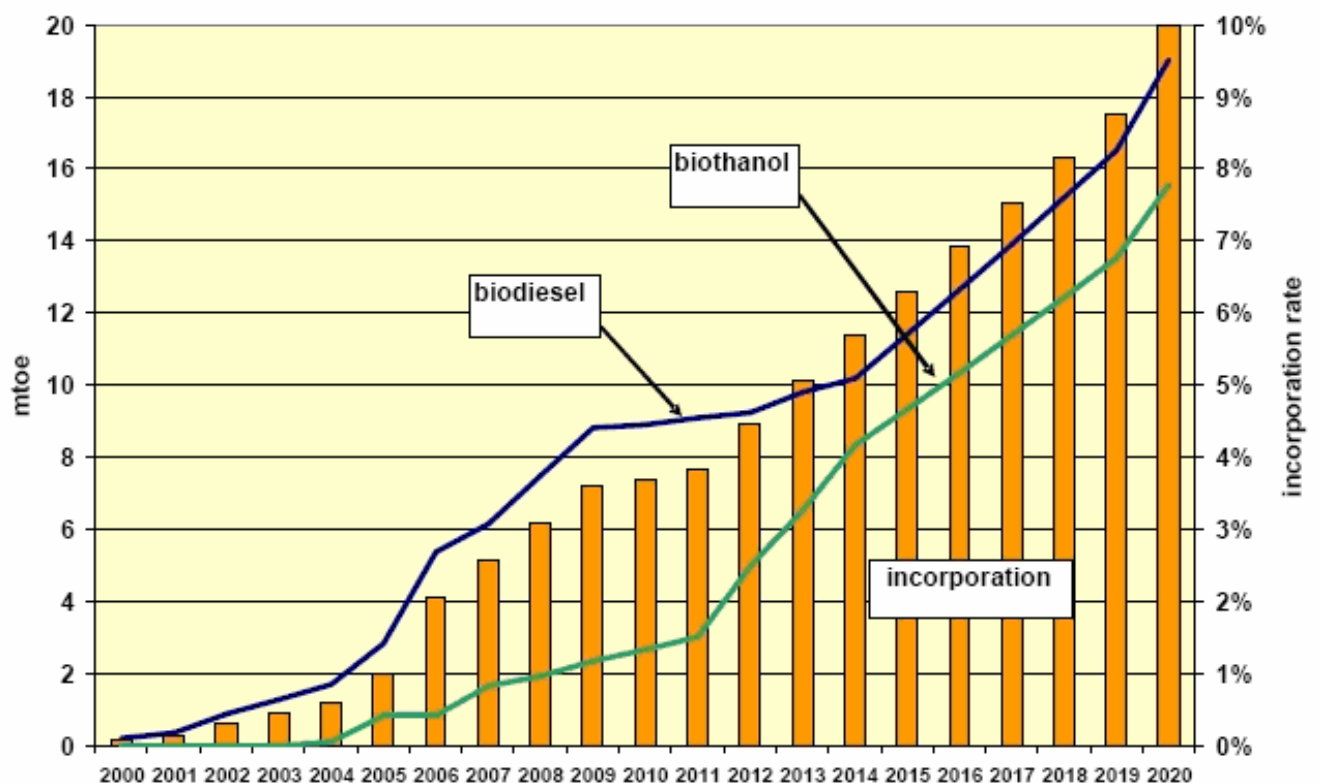


Figure 2. Illustrative development of biodiesel and bioethanol demand and the incorporation rate until 2020 in the EU-27. Adapted from [39]

There are different feedstocks for production of biofuels [4]. In North America, bioethanol is mostly produced from starch of grains, but grains are produced for food and animal feed. By increasing the bioethanol production from grains in the future, the grain will not cover the demand and this will increase the price of food which will leads to other problems, but Sweden is rich in wood based lignocellulosic materials which can be used for production of bioethanol.

A disadvantage connected to biofuels is their lower energy density compared to diesel and gasoline. More than a liter of bioethanol is necessary to replace a liter of diesel or gasoline [5]. Question is the cost of bioethanol production; the production

cost must be very low to be able to be competitive with gasoline. The cost of raw material has a great impact on the cost of bioethanol production; lignocellulosic materials are relatively cheap and plentiful and considered to be the main source of feedstocks for low-cost bio-ethanol production [6] and it is believed that future progress in biotechnology will decrease the cost of bioethanol production.

1.3. The composition of Lignocelluloses

Lignocelluloses consist of three main compounds: cellulose, hemicellulose and lignin. Compositions of cell wall of the woods are influenced by genetic variation of species, growth conditions and age of the plant. The composition and structure of the woods are important in production process. Hardwoods (e.g. alder, aspen and birch) and softwoods (e.g. pine and spruce) contain an average of 42% cellulose, 20% hemicellulose and 21% lignin [7] and Extractives. Hardwood cellulose is rich in xylans and contain limited amount of mannans whereas softwood hemicelluloses are rich in mannans and contain low amount of xylans. Softwoods generally contain higher amounts of lignin than hardwoods. Due to their structural differences the chemical compositions of the woods dilute acid hydrolysates are different [8].

1.3.1. Cellulose

Cellulose is an organic compound with the formula $(C_6H_{12}O_6)_n$. It is the primary structural component of green plants.

Cellulose is repeating units of cellobiose, $C_{12}H_{22}O_{11}$, which consists of two units of D-glucose, linked together by β -1 \rightarrow 4 linkages (Figure 3). The β -linkages form linear chains are resistant to chemical attack because of the hydrogen bonds that can occur between the chains of cellulose. That is the reason of why cellulose needs a harder condition of hydrolyses. Hydrolysis will reduce cellulose to cellobiose and ultimately to glucose.

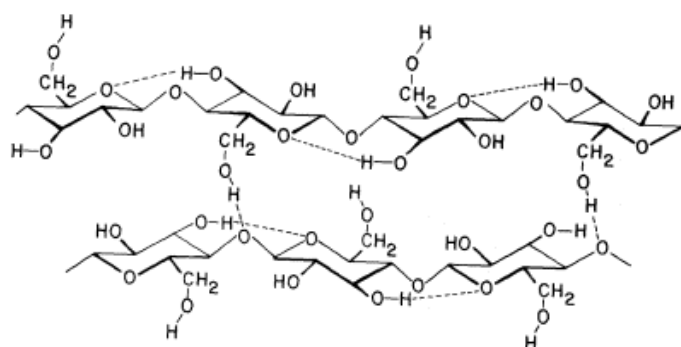


Figure 3.b-1,4linked glucan chains of cellulose.
Portions of two associated chains are illustrated by conformational line drawings.
Adapted from [9]

1.3.2. Hemicellulose

It is easier to hydrolyse hemicelluloses by dilute acid or base into its monomers because of its non crystalline structure. Hemicellulose are branched polymers and consist of shorter chain than cellulose and contains many different sugar monomers , mainly D- sugars such as D-galactose (hexsose), D-glucose(hexsose), D-mannose (hexsose), D-xylose (phentose) and small amounts of L-sugars like L-arabinose (phentose), (figure 4).

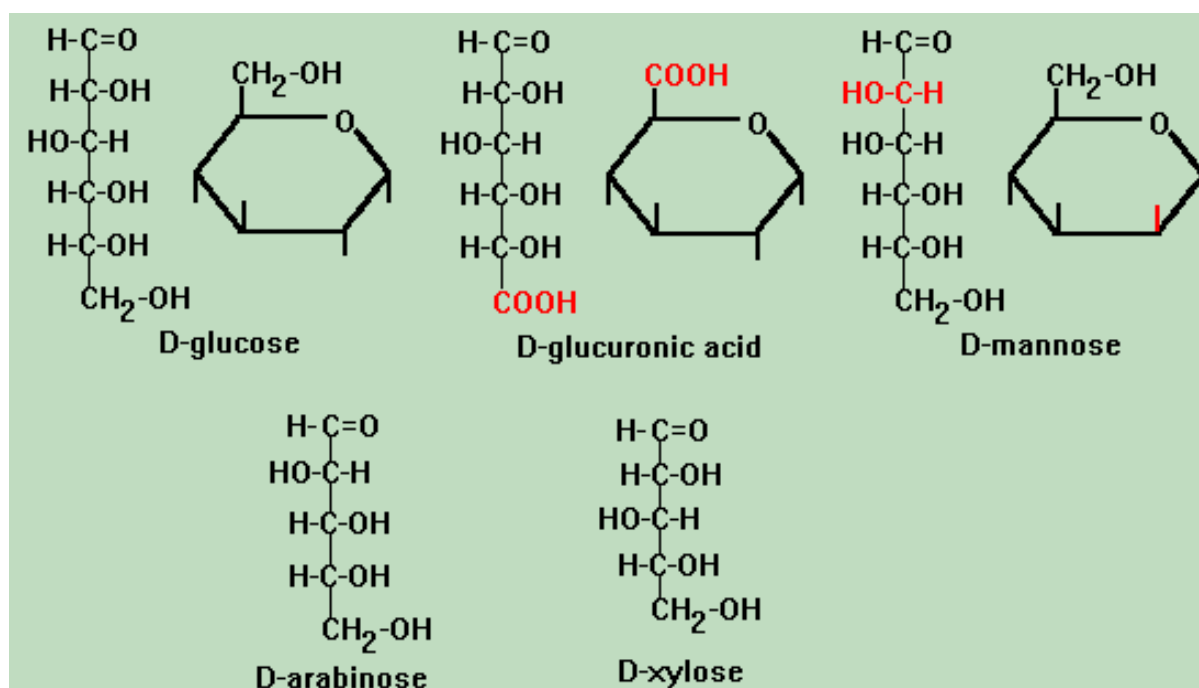


Figure 4. D-pentose sugars such as D-galactose, D-glucose, D-mannose, D-xylose the groups that are different from glucose are shown in red on the six-carbon structures. Adapted from [10]

1.3.3. Lignin

Lignin is a complex polymer of phenylpropane units (Figure 5), which are cross-linked to each other with different chemical bonds and it is one of the most slowly decomposing components.

In the cell wall, lignin fills the spaces between cellulose and hemicellulose mostly by hydrogen bonds but also by covalent bonds. Due to these bonds it is especially resistant to chemical attack or enzymatic degradation but there are some fungi and bacteria that are able to biodegrade lignin. Lignin degradation is an aerobic process; in an anaerobic environment lignin can persist for long time [11]. Lignin yields more energy when it burns than cellulose therefore it is a good raw material for production of fuel.

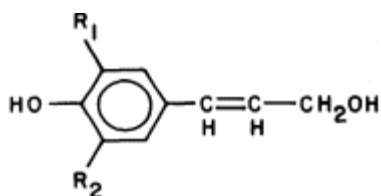


Figure 5. Lignin. Adapted from the book; [12]

1.3.4. Extractives

Extractives are a group of chemicals in the cell wall which mainly consist of fats, fatty acids, fatty Alcohols, phenols, terpenes, steroids, resin acids, rosin, waxes and many other minor organic compounds. These chemicals exist in their monomers, dimmers or polymers forms. In softwoods, the content of extractives are higher than hardwoods [13].

Extractives are chemicals in the wood that can be extracted using solvents. The extractives are classified by the solvent used to extract them, for example, water-soluble or toluene-ethanol-soluble or ether-soluble extractives [13].

1.4. Bio-ethanol production from lignocelluloses

Ethanol can be produced from lignocellulose materials in different ways. The general procedure to convert lignocellulose material to bioethanol is hydrolysis of the hemicellulose and the cellulose to its monomer sugars, fermentation, and distillation. Hydrolysis can be either enzymatic or chemical. In chemical hydrolysis, acids such as sulphuric (cheap and effective), hydrochloric, nitric and formic acid can be used to break down cellulose and hemicellulose to its monomers. The concentrated acid hydrolysis makes possible to use lower temperature which reduce the by-products amount and gives higher sugar yield. Although the sugar yields are lower when dilute-acid is used, it is preferred due to easier acid-recovery, low acid consumption and lees equipment corrosion which reduce the production cost. The hydrolysis will carry out in two steps because of the different structures of cellulose and hemicellulose. In the first stage, hemicellulose is hydrolysed under milder conditions (170 –190 °C), whereas in the second stage cellulose is hydrolysed under harsher conditions (200-230 °C) [14]. This two-stage process helps the hemicellulose sugars to not be converted to degradation products such as furfural 5-hydroxymethylfurfural (HMF), phenolic compounds (phenol, formaldehyde) and weak carboxylic acids such as acetic acid and formic acid which cause problems in fermentation stage due to their inhibitory effects to microorganisms, which leads to lower ethanol production.

An alternative to chemical hydrolysis is enzymatic hydrolysis which needs an extra pre-treatment, heat, and acidic condition to make the cellulose fibers easily available to the enzymes. Most of the hemicelluloses are broken down in the pre-treatment step, the cellulose enzymes that are needed to decompose cellulose are a mixture of three classes of enzymes: exo-1,4-β-D cellobiohydrolases, 1,4-β-D-glucosidases and

endo-1,4- β -D-glucanases [15]. Disadvantage of enzymatic hydrolysis is the slow hydrolysis rate and the expensive cost of the enzymes.

1.4.1. Product formation in dilute acid hydrolysis

In dilute acid hydrolysis, of lignocellulose materials, the hemicelluloses are broken down in the first step with milder condition into hexoses (glucose, galactose and mannose), pentoses (xylose and arabinose) and acetic acid which is released from chemical combination due to hemicellulose sugars are acetylated [17] [18].

In the second step with higher temperature and longer residence time, cellulose is converted to glucose. This high temperature causes the break down of released sugars and produce decomposition products such as furfural from pentoses and 5-hydroxymethylfurfural (HMF) from hexoses. 5-hydroxymethylfurfural (HMF) is further broken down to levulinic acid and formic acids [16] while furfural will be broken down to e.g. formic acids.

Minor parts of lignin and extractives are also degraded to phenolic components. It has been shown that different phenolic compounds are formed during acidic condition and heating from glucose, xylose and arabinose [17] [18].

1.5. Fermentation of hydrolysates

There are three operation methodologies for fermentation: Batch, fed-batch and continuous fermentation.

1.5.1. Batch fermentation

Batch culture can be considered as a closed culture system which contains an initial, limited amount of nutrient, which is inoculated with microorganisms to allow the fermentation. It is very simple method, during the fermentation nothing is added, except oxygen (if it is an aerobic fermentation), antifoam and acid or base to adjust the pH. During the process, the composition of the culture medium, biomass concentration and metabolite concentration change constantly. This method is usually used in laboratories and for food and pharmaceutical industries because the risk of contamination in batch methodology is small due to its easy sterilization process [19].

After inoculation, there is a period in which no growth takes place. It is considered as a time of adaptation. This period is called for lag phase. In a commercial process, it is better to reduce the length of lag phase as much as possible. To minimize the lag phase period the inoculum development medium should be sufficiently similar to the production medium, major differences in pH and anion composition may change the uptake rates which, in turn affect the ability of growth [19].

After lag phase, there is a period in which nutrients are in excess and the organisms growing rate gradually increases (Figure 6). This period is known as exponential

phase or log phase. But after a certain time the growth rate decreases until growth stops. It might be due to depletion of one or some most important nutrients in the medium, the accumulation of some autotoxic product of the microorganism in the medium or a combination of both. The next phase in batch culture is known as stationary phase, in that point the growth rate has decreased to zero but the microorganism are still metabolically active and may produce products called secondary metabolites, which are not produced during the exponential phase [20]. The length of time between the phases is dependent on the microorganism and the process used. The fermentation usually is stopped at the end of the log phase or before the death phase begins.

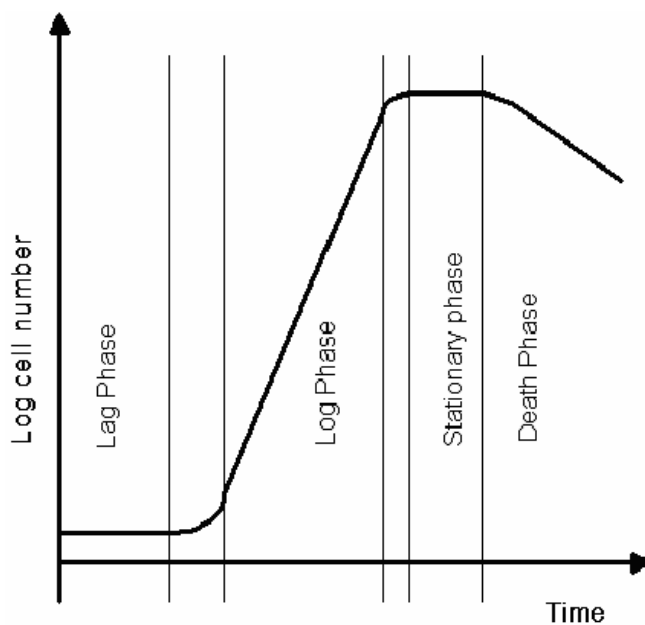


Figure 6. Adapted from [49]

Batch cultivation is not suitable for dilute-acid hydrolysate, unless the hydrolysate is detoxified and the inoculum size is high. It was shown that a combination of low concentration of cells and high concentration of inhibitors, inactivate the cells and prevent their growth [55].

1.5.2. Fed-batch fermentation

In a fed-batch system, the essential elements of the nutrient solution are added in small concentrations at the beginning of the process. But opposite to the batch cultivation system, these substances continue to be added in small doses during the fermentation.

Today, fed-batch methodology is used for the production of antibiotics, amino acids, vitamins, enzymes and growth hormones. Fed-batch technique has been classified in two main groups; those with feedback control and those without feedback control of lignocellulosic hydrolysates [21].

The advantages of fed-batch culture are: with respect to fermentation it increases the production of biomass (no washout cells), reduce broth viscosity and overcome the problems of contamination and mutations which are found in continuous culture [21]. The disadvantages of this method are: it is costly due to additional instruments for feedback control and needs good skill operator [21].

Fed-batch technique is a suitable method to ferment dilute-acid hydrolysate. The ethanol productivity is higher in fed-batch fermentation compared to batch fermentation because of high concentration of inhibitors that can be avoided in fed-batch fermentation [22].

1.5.3. Continuous fermentation

Continuous culture methodology have been used in industrial scale for over 50 years such as vinegar production, waste treatment and yeast propagation (reproduce organism). In laboratory-scale, this method is useful for studying the physiology of microorganisms and their growth [21].

Continuous fermentation is an open system; sterile nutrient solution is added to the system continuously while an equal amount of converted nutrient solution with microorganisms is taken out of the system. If medium is fed continuously at a suitable rate, a steady state condition is obtained, that means formation of new biomass is almost equal to the loss of the cells from the vessel [21].

Continuous fermentation has an advantage compared to batch cultivation regarding fermentation of diluted-acid hydrolysates. In the continuous cultivation the concentrations of the convertible inhibitors in the medium are kept low and because of that continuous cultivation has higher potential for *in situ* detoxification over batch cultivation.

1.6. Microorganism

Microorganisms play a great role in production of ethanol from renewable resources and selection of suitable strain is necessarily for individual process.

Capability of consumption both pentose and hexose sugars, high tolerance against substrate, ethanol as well as inhibiting compounds, high ethanol yield and minimum nutrient requirements are the important facts of an ideal microorganism [45].

Bacteria, yeasts and filamentous fungi are able to ferment hydrolysates from different plants.

1.6.1. Bacteria

Escherichia coli have the ability of utilizing glucose, mannose, xylose and arabinose. Its ethanol tolerance is 50g/l [23], but the ethanol yield of this bacterium is low. A result to this problem is genetically modification, by inserting *Zymomonas mobilis*

genes a recombinant strain of *E.coli* KO11 were produced, it was able to produce ethanol from corn fibre hemicellulose hydrolysate with yield of 0,41 g/g with a productivity of 1,16 g/l·h [24].

Zymomonas mobilis is another bacterium which is very similar to *Saccharomyces cerevisiae*, but this organism showed to be sensitive to acetic acid [25], which is always present in lignocellulosic hydrolysates.

1.6.2. Yeast

Due to the great properties, *Saccharomyces cerevisiae* is widely used for fermentation of hydrolysates to produce ethanol. *S.cerevisiae*'s suitable properties for fermentation of hydrolysates are high ethanol yield, high ethanol productivity, high ethanol tolerance and it's tolerance to low pH [26]. But *S.cerevisiae* has a limitation, it can not utilize xylose because it does not have genes encoded for xylose reductase (XR) and xylitol dehydrogenase (XDH) [27]. The fermentation of xylose is very important due to the high contain of D-xylose in the hydrolysates from hardwoods, softwoods and agricultural residues such as sugarcane bagasse, corn stove, wheat straw and barley straw that are potential feedstocks for ethanol production [28] (Table1). To improve the economics of biomass conversion to ethanol, it is very important that the micoorganisms are able to convert efficiently both hexoses and pentoses to ethanol. In order to reach that purpose, there have been intensive efforts to produce wild type organisms which are able to produce ethanol anaerobically from xylose with high yield.

Table 1. Feedstock's for bioethanol and the contain of D-xylose

Feedstock for bioethanol	D-xylose
Hydrolyses from hardwood	20-34% xylose
Hydrolyses from soft wood	Up to 13%
Agricultural residues	Are also rich in-xylose

1.6.3. Filamentous fungi

Zygomycetes are filamentous fungi that have the ability to utilize several different kind of sugars as glucose, mannose, galactose, xylose, arabinose, cellobiose [30,31]. Below, seven zygomycetes strains belonging to the *Rhizopus*, *Mucore* and also *S.cerevisiae* are compared in terms of sugars consumption and ethanol production in aerobic cultivation in a synthetic medium with 50 g/l glucose and 50g/l xylose in 30°C for 36 hours, except *M.circinelloides*, which were cultivated in 20 g/l xylose and glucose for 72 hours.

Table 2. Ethanol productivities, ethanol and biomass yields of different zygomycetes strain in aerobic cultivation on 50 g/l glucose except *M.circinelloides* which were cultivated in 20 g/l glucose.

No	Strain	Glucose (g/l)	Ethanol productivity (g/l)	Y _{Ethanol/S} (g/g)	Y _{Biomass/S} (g/g)	Ref.
	<i>Rhizopus</i>					
1.	<i>R.oryzae</i> CCUG 28958	50	1,87	0,41	0,11	[32]
2.	<i>R.oryzae</i> CCUG 22420	50	1,00	0,43	0,13	[32]
3.	<i>R.oryzae</i> CCUG 18663	50	1,24	0,37	0,07	[32]
	<i>Mucor</i>					
4.	<i>M.corticolous</i> CCUG 0481	50	1,48	0,43	0,05	[32]
5.	<i>M.hiemalis</i> CCUG 16178	50	1,44	0,39	0,09	[32]
6.	<i>M.indicus</i> CCUG 22424	50	1,41	0,39	0,12	[32]
	<i>M.circinelloides</i> ATCC 1216B ^a	20	Not reported	0,34	0,31	[56]
	yeast					
7.	<i>S.cerevisiae</i>	50	1,29	0,42	0,05	[32]

^a cultivated in 20 g/l glucose

Table 3. Ethanol productivities, ethanol and biomass yields of different zygomycetes strain in aerobic cultivation on 50 g/l xylose, except *M.circinelloides* which were cultivated in 20 g/l xylose

No	Strain	xylose (g/l)	Ethanol productivity (g/l)	Y _{Ethanol/S} (g/g)	Y _{Biomass/S} (g/g)	Ref.
	<i>Rhizopus</i>					
1.	<i>R.oryzae</i> CCUG 28958	50	0,07	0,16	0,17	[32]
2.	<i>R.oryzae</i> CCUG 22420	50	0,11	0,28	0,17	[32]
3.	<i>R.oryzae</i> CCUG 18663	50	0,11	0,23	0,09	[32]
	<i>Mucor</i>					
4.	<i>M.corticolous</i> CCUG 0481	50	0,10	0,15	0,13	[32]
5.	<i>M.hiemalis</i> CCUG 16178	50	0,12	0,18	0,11	[32]
6.	<i>M.indicus</i> CCUG 22424	50	0,18	0,22	0,12	[32]
7.	<i>M.circinelloides</i> ATCC 1216B ^a	20	Not reported	0,004	0,61	[56]

^a cultivated in 20 g/l xylose

The results in Table 2 and Table 3 show that all the *Mucor* and *Rhizopus* strains were able to utilize glucose and xylose to produce ethanol, the ethanol productivity of all the *Mucor* strains and two strains of *Rhizopus* from glucose was over 1,2 g/l·h but the ethanol productivity from xylose of these strains were lower. The ethanol productivity, of *M.indicus* in aerobic cultivation on 50 g/l xylose was 0,18 g/l which is higher than the other seven zygomycetes strains [32].

It is also shown in previous experiments that all three mentioned *Mucor* strains showed high ethanol productivity from dilute-acid hydrolysate and they also produce negligible amounts of lactic acid from glucose. The faster ethanol producer from xylose amount the tested strains was *M.indicus* [32].

1.7. *Mucor indicus (rouxii)*

Mucor is a filamentous fungus. It is found in solid, plants, rotten fruits and vegetables. The colour is white, becomes greyish-brown by time. It grows rapidly at 25-30 °C but it can even grow in up to 40 °C. *M.indicus* is a safe zygomycete for humans. It is able to utilize a wide range of hexoses, pentoses (monosaccharide) [35] and disaccharides (cellobiose) [36]. The ethanol yield and productivity of this microorganism from hexoses are as high as *Saccharomyces cerevisiae*, and it is also able to utilize xylose [32-33]. That is the reason of why it is one of the candidates for ethanol production. Another advantage is the biomass of this fungus, since it contains relatively high concentrations of chitosan, which is useful in many applications [34].

But still fermentation of lignocellulosic hydrolyzates by *M. indicus* has not been industrially reported yet [32-34].

Table 4. The classification of *M.indicus*

Kingdom	Fungi
Phylum	Zygomycota
Class	Zygomycetes
Order	Mucorales
Family	Mucoraceae
Genus	Mucor
Species	Indicus

1.7.1. Morphology

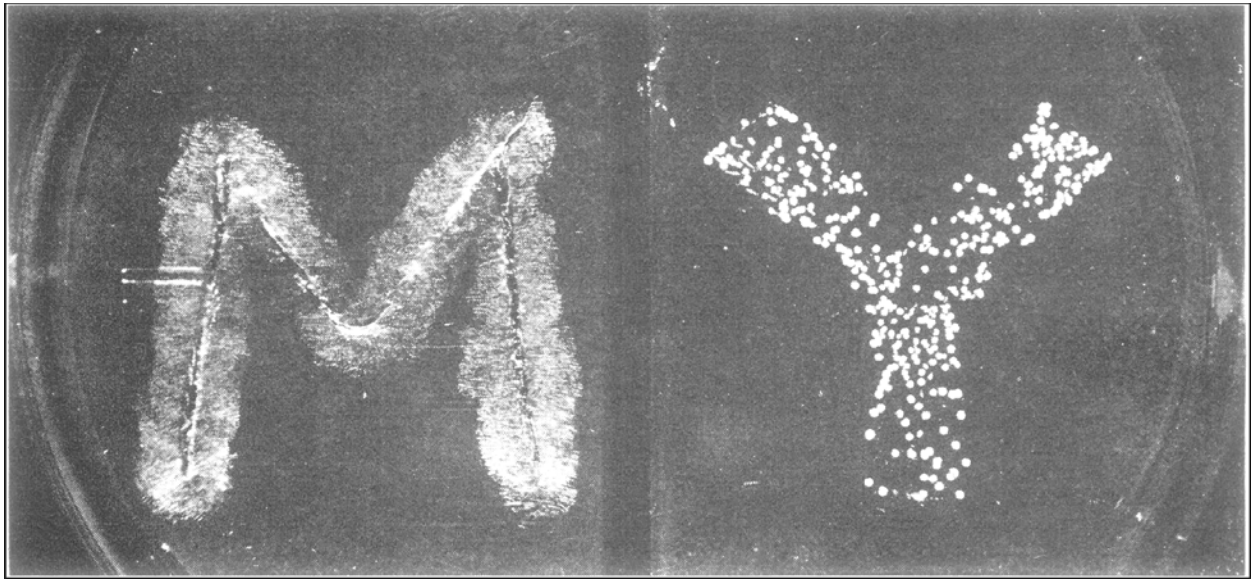


Figure 7. Control of dimorphism by glucose concentration. Agar plate under 30% CO₂- (left) Mycelium on 0,05% glucose; (right) yeast colonies on 5% glucose. The plate was incubated for 30 hr. (left) Spores were inoculated in a single M-shaped streak; (right) spores were deposited on the full width of the Y area. Adapted from [50]

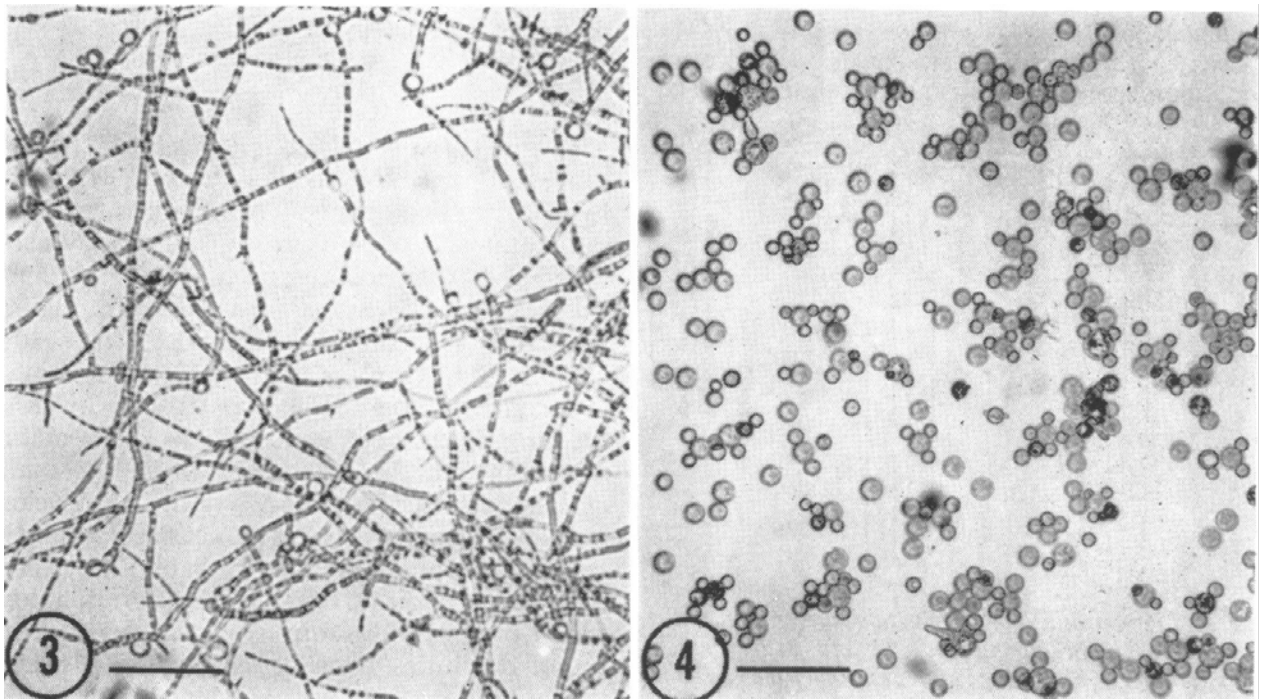


Figure 8. Control of dimorphism by glucose concentration. Liquid cultures under 30% CO₂. (left) Hyphae in 0,01% glucose; (right) yeast cells in 1% glucose. Incubation time was 24 hr. Magnification marker = 100 Am. Adapted from; [50]

Various species of *Mucor* have capacity to grow as a mycelium or yeast-like. There are many parameters that control morphology in a fermentation process such as size

of inoculums, type and amount of carbon sources, pH of medium and type of nitrogen source[37], (Table 5).

Table 5. Differences between microscopic and macroscopic growth patterns. Adapted from: Ethanol production from lignocellulosic materials: Potential of continuous cultivation, Immobilisation and Zygomycetous fungi . By Ria Millati

Microscopic dimorphism	Macroscopic growth forms
Yest-like Filamentous hyphae	Suspension Cotton-like or Pellets

The dimorphic growth of *Mucor rouxii* is reversible. It means if aerobically grown mycelium is transferred to a carbon dioxide atmosphere, it starts to grow yeast-like. On the other hand, if anaerobically grown yeast populations transferred to aerobic atmosphere, it starts growing mycelia form. The yeast cells, cell wall is thicker, less dense and chemically differs from the mycelia cell wall. The initial stages of spore development are independent of atmospheric composition until the spore wall is swollen; oxygen starts the mycelia life cycle and carbon dioxide in the absence of oxygen, starts the yeast life cycle [38].

Table 6. Growth and morphologies of species of *Mucor*. grown anaerobically in liquid YPG medium. Adapted from: [51].

Organism	Strain*	Anaerobic atmosphere†			
		N ₂		CO ₂	
		Growth	Morphology	Growth	Morphology
<i>M. rouxii</i>	ATCC 4855	++	MF	+++	MY
<i>M. rouxii</i>	ATCC 5857	++	F	++	MY
<i>M. rouxii</i>	ATCC 8097	++	MF	+++	Y
<i>M. rouxii</i>	CBS	++	MF	++	MY
<i>M. rouxii</i>	IM 80	++	MF	+++	Y
<i>M. subtilissimus</i>	NRRL 1909	+++	Y	+++	Y
<i>M. subtilissimus</i>	NRRL 1743	++	MF	+++	Y
<i>M. racemosus</i> (+).....	NRRL 1427	+	F	+	MF
<i>M. racemosus</i> (–).....	NRRL 1428	++	MF	+	Y
<i>M. rammanianus</i>	IM 93	Not tested		0	—
<i>M. mucedo</i>	DC	Not tested		+++	MY
<i>Mucor</i> sp.....	DU	Not tested		++	Y

* ATCC = American Type Culture Collection; CBS = Centraalbureau voor Schimmelcultures; IM = Institute of Microbiology, Rutgers University; NRRL = Northern Regional Research Laboratory, cultures received through the courtesy of C. W. Hesseltine; DC = Douglass College, Rutgers University; DU = Duke University School of Medicine.

in all cases, cultures incubated under air showed heavy filamentous growth, with little or no fragmentation. Amount of growth was estimated visually: 0 = no growth; +++ = abundant growth. Morphology of the cultures was classified as follows: F =

purely filamentous; MF = mostly filamentous, with some spherical cells; Y = purely yeast-like; MY = mostly yeast-like, with few filaments

Different *Mucor* species were cultivated on a complex culture medium (YPG) with 20 g/l glucose in a shaker at 28 °C for 48 hours. In order to provide anaerobic condition purified N₂ of 99,996 % purity or CO₂ of 99,8% purity were flushed through the gas space of flasks and to get an aerobic condition air were replaced with either N₂ or CO₂ [35].

Anaerobic incubation of *M.indicus* under atmosphere of N₂ results filamentous growth. The rate of growth and total amount of growth in the anaerobic incubation under N₂ atmosphere were less than the aerobically growth, but morphological features were similar. Anaerobic incubation under pure CO₂ result different appearance, the growth was yeast-like cells with no trace of filamentation. Aerobic growth was more abundant than anaerobic growth. For most species of *Mucor*, in order to get yeast-like cells we must have presence of carbon dioxide and absence of oxygen [35].

1.7.2. Other applications of *M.indicus*

The cell wall of *M.indicus* contains significant quantity of chitosan/chitin which can be easily extracted [39-40].

Chitin is the second most abundant biopolymer on earth and is found mainly in insects, marine diatoms, invertebrates, algae, fungi, and yeasts. Chitosan is the deacetylated product of chitin. This process called deacetylation. They have many applications in chemistry, biotechnology, medicine, veterinary, dentistry, agriculture, food processing, environmental protection, water purification, cosmetic and textile industries [47].

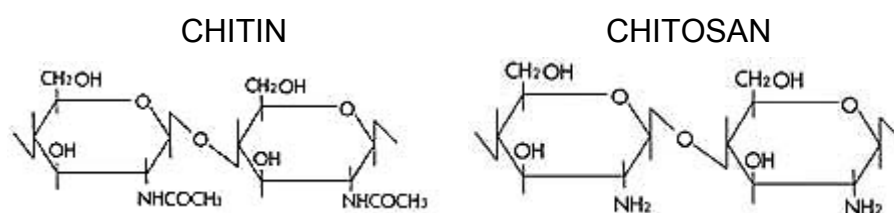


Figure 9. Chitin and chitosan. Adapted from: [52]

Some fungi such as *Mucor*, *Rhizopus* and *Absidia* species have chitosan as one of the structural components in their cell wall. In fungi, chitin exists in the cell wall of spores and hyphae and provides the framework in cell wall morphology [41]. The quantities of chitin and chitosan in mycelia *M.rouxii* can reach 35% of cell wall dry weight.

Table 7. *Several applications of chitosan* [42, 53, 54].

No.	Field	Application
1	Agriculture	Seed coating Fertilizer Antimicrobial activity
2	Food processing	Preservatives Colour stabilization cholesterol reduction
3	Chemistry	Thickening and gelling agents Cross-linking reactions
4	Textile	Textile finishing Textile auxiliary
5	Pharmaceutical and medical	Drug delivery Vaccination
6	Waste water purification	Flocculation Removal of metal ions
7	Biotechnology	Enzyme immobilization Protein separation Cell recovery Chromatography Cell immobilization
8	Cosmetics	Moisturizer Face , hand and body creams Bath lotion

1.8. Aerobic, anaerobic ethanol production by *M.indicus*

In the aerobic condition on synthetic medium as well as wood hydrolysates, *M.indicus* is able to utilize a large variety of carbon sources, including hexoses (glucose, mannose and galactose) and xylose. The fungus is able to consume xylose after complete consumption of hexoses [44].

In the anaerobic fermentation, the ethanol yield and biomass yield by *M.indicus* on glucose were 0,46 g/g and 0,6 g/g [33]. The ethanol yield of cultivation on diluted-acid hydrolysate was 0,44 g/g [43].

The ethanol yield from xylose in aerobic cultivation was 0,18 g/g [33]. Xylose was consumed more quickly at higher aeration rate and in complete absence of oxygen the consumption of xylose was not possible [33,57].

The aerobic cultivation of *M.indicus* compared to anaerobic cultivation gives lower ethanol yield but higher biomass yield (0,40 g/g and 0,16 g/g) [33], due to the ability of cells to consume ethanol under aerobic fermentation. During both aerobic and anaerobic cultivation on glucose, the most abundant metabolite is ethanol. Glycerol is the main by-product and some carboxylic acids such as acetic, pyruvic and succinic are also produced. Xylitol is also detected when the medium contained xylose [43].

Table 8. The ethanol and biomass yields of *M.indicus* CCUG22424 in aerobic and anaerobic cultivation on synthetic medium of 50 g/l glucose.

Strain	Sugar	Aerobic		Anaerobic	
		YEthanol/S (g/g)	YBiomass/S (g/g)	YEthanol/S (g/g)	YBiomass/S (g/g)
<i>Mucor indicus</i>	glucose	0,40	0,16	0,46	0,06

1.9. The aims of this project

The main goal of this research was to investigate the ethanol tolerance and glucose tolerance of *M.indicus* in aerobic and anaerobic conditions. Study of glucose tolerance was performed on synthetic medium with different concentrations of glucose under aerobic and anaerobic cultivations. Study of ethanol tolerance was performed on synthetic medium with D-glucose as carbon and energy source with different concentrations of ethanol.

In addition to the experiments above; anaerobic cultivations by *M.indicus* CCUG22424 on synthetic medium were carried out to study sugars consumption and ethanol production of *M.indicus* on three different kinds of sugars, D-glucose, D-xylose and xylitol (10 g/l) as carbon and energy source. Furfural or HMF was added after 13 hours of cultivation on 10 g/l of xylose to find out their individual effect as an electron-acceptor.

2. Materials and methods

2.1. The microorganism

The microorganism used through out the experiments was *Mucore indicus* CCUG22424, which was obtained from Culture Collection, University of Göteborg, Sweden. The strain was maintained at pH 5,6 on potato dextrose agar slants made from 10 g/l neopeptone (Difco, Sparks ,MD, USA), 15 g/l agar and 40 g/l D-glucose as additional carbon source, incubated for four days at 30 °C. The slants were stored at 4 °C before use or it can be stored at 4 °C for one year (less than 4 °C will kill a great amount of the microorganism).

2.2. Cultivation

Aerobic cultivations were performed in 300 ml-Erlenmeyer flasks closed with cotton-plugged, while anaerobic cultivations were performed in 300 ml-Erlenmeyer flasks closed with loop trap. The loop trap contained 2 ml autoclaved glycerol to prevent air diffusion into the flasks, but the released gas from fermentation was able to pass through the glycerol into the air.

The medium of all experiments consisting of different concentrations of glucose supplemented with (g/l): yeast extraction, 5; (NH₄)₂SO₄, 7,5; MgSO₄ · 7H₂O, 0,75; KH₂PO₄, 3,5; CaCl₂ · 2H₂O, 1, at pH 5,5± 0,1. Just in experiment “ethanol tolerance of *M.indicus* in anaerobic condition” instead of CaCl₂ · 2H₂O, 1 g/l, Trace metal solution 10 ml/l were added.

Sugars were autoclaved separately from the medium, and mixed afterwards. For inoculation, a slant with *Mucor indicus* was dissolved in 10 ml of dionized water, and 3 ml of this solution was added into each flask. The total volume of each flask was 153 ml. The flasks were kept in a shaker bath at 30 °C with a shaker speed of 150 rpm.

In the aerobic cultivation of “Glucose tolerance of *M.indicus*” the experiment was carried out in 10 shake flasks with different concentrations of D-glucose (30, 60, 85, 100, 190, 200, 250, 300, 350, 400 g/l) as carbon source and in anaerobic cultivation of “Glucose tolerance of *M.indicus*” the concentrations of D-glucose were (30, 70, 100, 150, 200, 250, 300, 350 g/l).

In the anaerobic cultivation of “Ethanol tolerance of *M.indicus*” the experiment was carried out in 6 shake flasks with 30 g/l D-glucose as the carbon source. 14 hours after cultivation 50 g/l autoclaved glucose and at the same time different concentrations (20, 40, 65, 80, 120, 180 g/l) of pure ethanol (99 –100%) were added to the flask. The total volume of each flask was 153 ml.

In the anaerobic cultivation of “Ability of *M.indicus* to consume different sugars” the experiment was carried out in 5 shake flasks with 10 g/l sugars (1x glucose, 1x xylitol,

and 3x xylose) as the carbon source. After 12 hours in one of the xylose flask 1,2 g/l 5-hydroxymethylfurfural(HMF) and in the second xylose flask 3,5 g/l furfural were added as an electron acceptors.

For certain time, sample of 4 ml was taken, centrifuged for 4-8 min with a speed of 5000 rpm and 2 x 1 ml were stored in a freeze -20°C . The rest of the sample was used to measuring the pH of the solution. During the experiments, the pH was adjusted to 3,5 – 4,5 by adding 2 M. NaOH. In order to have an anaerobic condition, pure nitrogen was sparged through a sterile filter for 2 minutes after taking each sample.

Table 9. The Composition of trace metal solution. The compounds in table below were dissolved in dionized water, the pH were adjusted to 4 by NaOH and autoclaved before using it.

Compound	Concentration (g/l)
Trace metal solution	
EDTA	3
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0,9
$\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$	0,9
$\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$	0,6
H_3BO_3	0,2
$\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$	0,15
$\text{Na}_2 \text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0,08
$\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$	0,06
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0,06
KI	0,02

2.3. Analytical methods

The metabolites were analyzed by high-performance liquid chromatography (HPLC). Glucose, xylose, xylitol, ethanol, glycerol, furfural, hydroxymethylfurfural (HMF), acetic acid and succinic acid were analyzed by an Aminex HPX87H column (Bio-Red, USA) at 60°C with a flow rate of 0,5 ml/min. The elluent was 5 mM H_2SO_4 (H_2SO_4 were diluted in ultra-pure water, Mili-Q water). Two detectors were used in series, a refractive index (RI) detector (Waters 410, Millipore, Milford; CA,USA) and UV absorbance detector at 210 nm (Waters486) were used. Concentrations of all metabolites were determined from RI chromatograms except furfural and HMF, which were determined from UV chromatograms.

2.4. Biomass determination

The biomass of each Erlenmeyer flasks was measured at the end of the experiment. The content of all flasks was collected separately and dried for 24 hours at 103 ± 3

°C. The biomass yield was calculated based on the total formation of biomass and the consumption of sugars.

3. Results

3.1. Glucose tolerance of *M.indicus* in aerobic cultivation

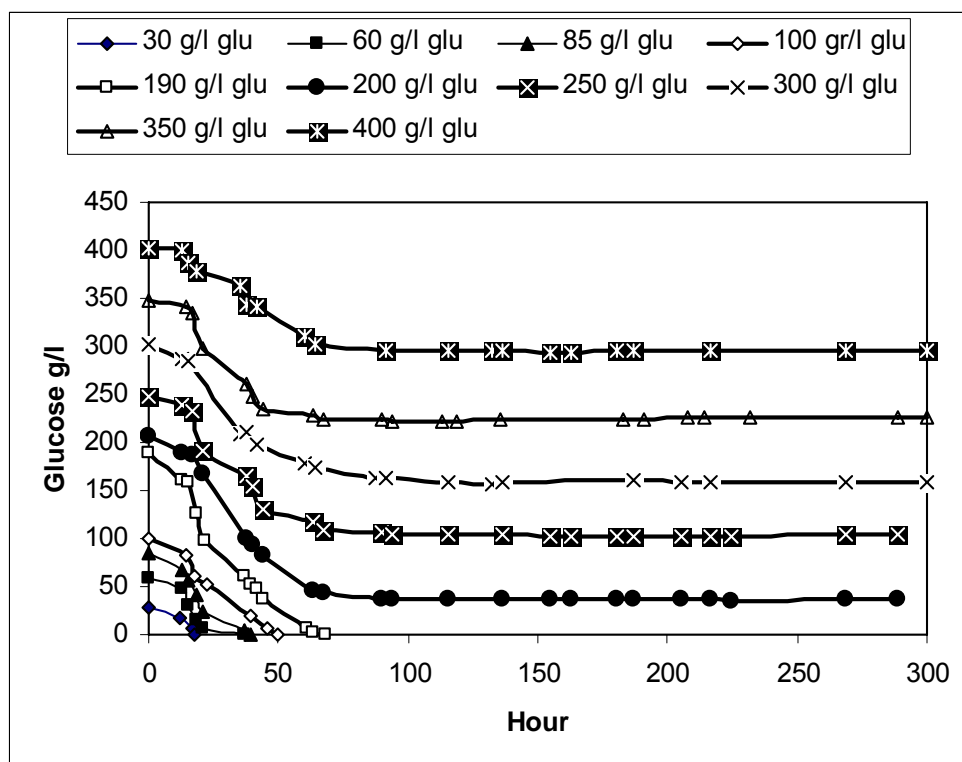


Figure 10. Glucose consumption of *M.indicus* in aerobic cultivation

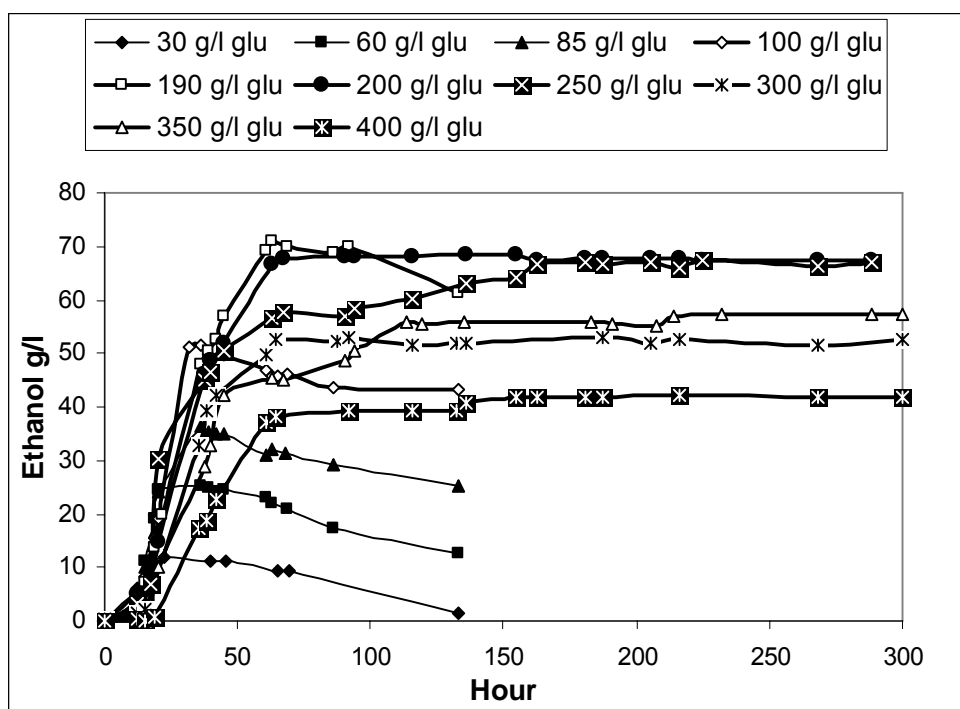


Figure 11. Ethanol production of *M.indicus* in aerobic cultivation

Table10. Total glucose consumption, total ethanol production, yields of biomass ethanol and glycerol, Max. glycerol, acetic acid, Succinic acid production and morphology of *M.indicus* CCUG2242 in aerobic cultivation on different concentration of glucose in a synthetic medium.

Initial glu. (g/l)	Total glu. consumption (g/l)	Max. eth. produced (g/l)	Eth. productivity (g/l)	Y _{eth./S} (g/g)	Y _{biomass/S} (g/g)	Max. gly. (g/l)	Y _{gly./S} (g/g)	Max. acetic acid (g/l)	Succinic acid (g/l)	Morphology
30	30	12	1,24	0,40	0,14	2	0,05	0,13	0,12	Cotton-like
60	60	25	2,49	0,41	0,09	5	0,06	0,85	0,17	Cotton-like
85	85	36	2,65	0,41	0,07	5	0,06	0,83	0,17	Cotton-like
100	100	43	1,53	0,41	0,05	7	0,07	0,91	–	Cotton-like
190	190	71	1,57	0,39	0,03	10	0,06	0,41	–	Cotton-like
200	170	68	0,70	0,40	0,03	10	0,07	0,49	0,45	Pellets and heavy suspension
250	145	67	0,65	0,40	0,03	12	0,08	0,57	0,39	Heavy suspension
300	144	51	0,61	0,36	0,03	13	0,09	0,67	0,42	Heavy suspension
350	125	47	0,21	0,34	0,02	15	0,12	0,70	0,40	Heavy suspension
400	106	42	0,38	0,34	0,02	22	0,21	0,94	0,11	Heavy suspension

3.2. Glucose tolerance of *M.indicus* in anaerobic cultivation

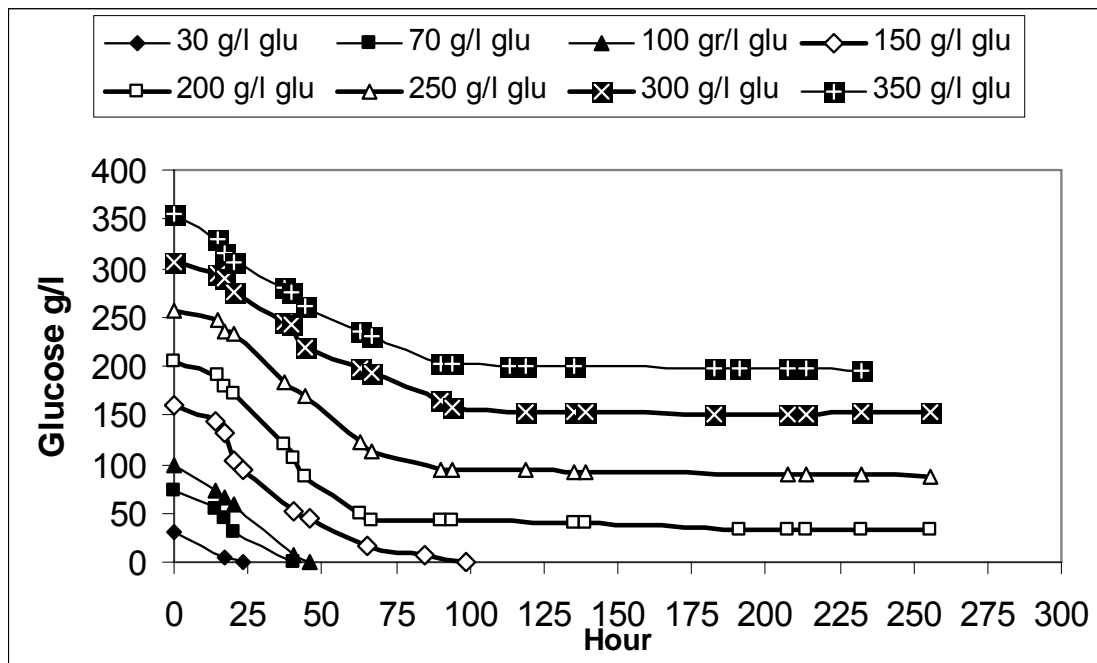


Figure12. Glucose consumption of *M.indicus* in anaerobic cultivation.

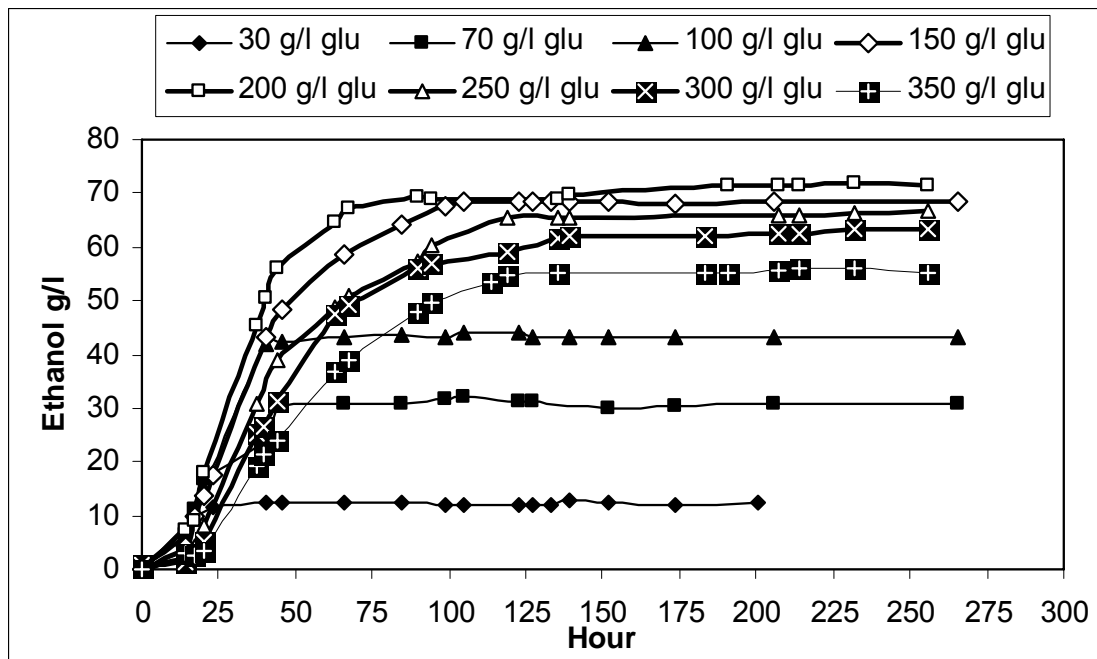


Figure13. Ethanol production of *M.indicus* in anaerobic cultivation

Table 11. Total glucose consumption, total ethanol production, yields of biomass ethanol and glycerol, Max. glycerol, acetic acid, Succinic acid production and morphology of *M.indicus* CCUG2242 in anaerobic cultivation on different concentration of glucose in a synthetic medium.

Initial glu. (g/l)	Total glu. consumption (g/l)	Max. eth. produced (g/l)	Eth. productivity (g/l)	Y eth./S (g/g)	Y biomass/S (g/g)	Max. gly. (g/l)	Y gly./S (g/g)	Max. acetic acid (g/l)	Succinic acid (g/l)	Morphology
30	30	12	0,56	0,41	0,06	2	0,068	0,14	0,10	Heavy suspension
70	70	32	1,75	0,41	0,03	6	0,078	—	0,19	Heavy suspension
100	100	44	1,56	0,43	0,03	7	0,075	0,35	0,28	Heavy suspension
150	150	65	1,27	0,42	0,03	11	0,068	0,11	0,37	Heavy suspension
200	172	73	0,7	0,42	0,02	22	0,139	0,11	0,39	Heavy suspension
250	167	68	0,6	0,40	0,02	18	0,11	0,16	0,39	Heavy suspension
300	155	63	0,5	0,40	0,02	19	0,12	0	0,40	Heavy suspension
350	156	54	0,5	0,35	0,02	20	0,12	0	0,65	Heavy suspension

Study of glucose tolerance was performed by cultivating strain *M.indicus* CCUG22424 on synthetic medium with different concentrations of glucose under aerobic and anaerobic cultivations. The effect of glucose concentration on glucose consumption, ethanol production, and growth are summarized in figures 10-13 and tables 10-11.

The results shows that the lag phase increased as the initial concentration of glucose increased. The fungus was able to grow, consume glucose and produce ethanol in all flasks in both aerobic and anaerobic conditions.

The results showed that the max. glucose consumption by this strain is between 190 to 200 g/l glucose in the aerobic cultivation. After consumption of the glucose, the ethanol concentration reached its peak in all flasks but than the amount of ethanol slowly starts to decrease, whereas in anaerobic cultivation the fungus was not able to utilize the produced ethanol. In anaerobic cultivations the total glucose consumption and ethanol production were higher compared to aerobic cultivations with the same concentration of initial glucose. It is easier to see this as the initial glucose concentration increase in the flasks. It is also showed that while the initial concentration of glucose increase the glucose consumption and ethanol production decrease, in both aerobic and anaerobic cultivations. The last 100 hours of cultivation only 1 to 2 g of glucose were consumed by *M.indicus* CCUG22424.

Glycerol was an important by-product in both aerobic and anaerobic cultivation in terms of concentration and yield. As initial concentration of glucose increased in the flasks, the production of glycerol also increased. In compared to aerobic cultivation the concentration of produced glycerol in anaerobic cultivation flasks with the same initial glucose concentration were a little higher.

Succinic acid and acetic acid are two other by-products. In compare to aerobic cultivation, the concentration of acetic acid which was produced during the cultivation was lower in anaerobic cultivation. During the aerobic cultivation the concentration of acetic acid is less than 0,1 g/l in all flasks, whereas the concentration of maximum succinic acid was lower than 0,3 g/l in all flasks in anaerobic cultivations.

Only one type of morphology, heavy suspension, was observed during the entire anaerobic cultivation with different concentration of initial glucose. But during the aerobic cultivation two different types of morphology cotton-like (or pellets) and heavy suspensions were observed. In aerobic cultivation cotton-like were mainly obtained in mediums with low concentration of initial glucose, whereas heavy suspensions obtained in mediums with high initial glucose concentration as carbon and energy source.

3.3. Ethanol tolerance of *M.indicus* in anaerobic cultivation

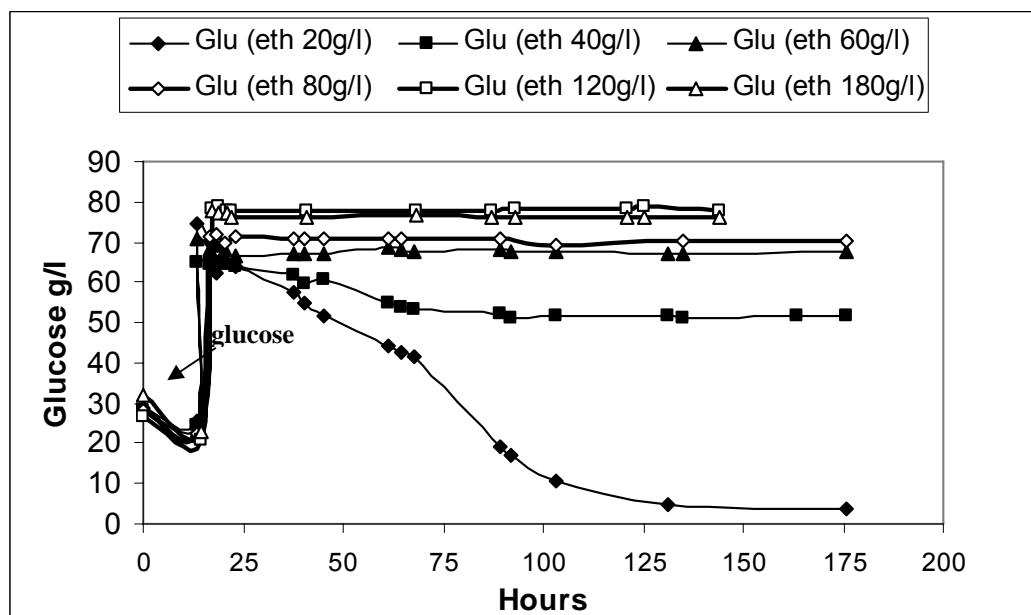


Figure 14. The capability of *M.indicus* CCUG22424 to consume glucose in different concentrations of ethanol.

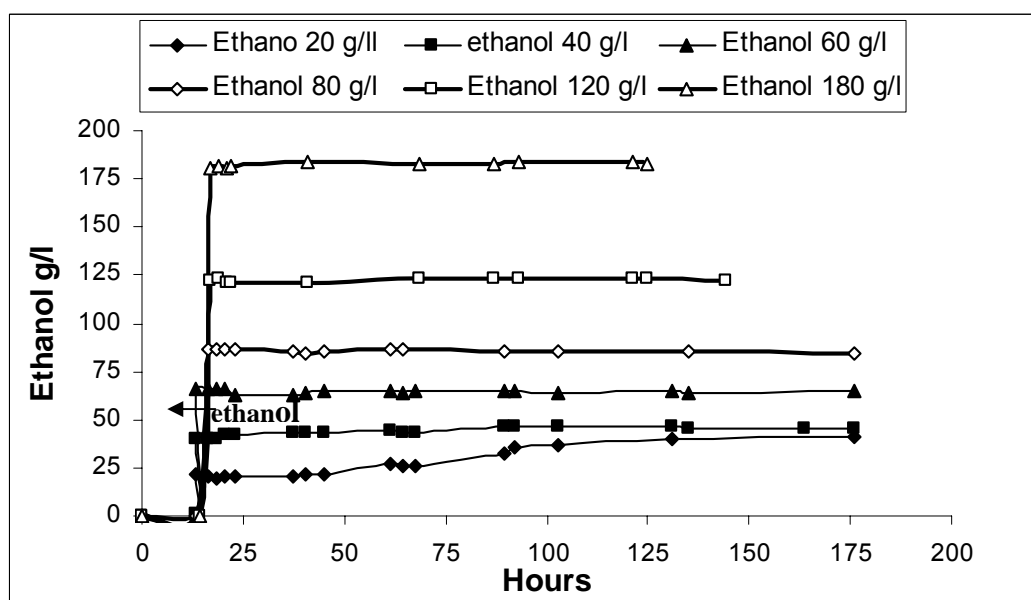


Figure 15. The capability of *M.indicus* CCUG22424 to produce ethanol in different concentrations of ethanol.

Table 12. Total glucose consumption, max. ethanol production, ethanol productivity, yields of ethanol and biomass and morphology of *M.indicus* CCUG2242 in anaerobic cultivation on different concentration of ethanol in a synthetic medium.

Initial Ethanol (g/l)	initial glucose	Total glucose consumption (g/l)	Max. ethanol produced (g/l)	Ethanol productivity (g/l)	Y Ethanol/S (g/g)	Y Biomass/S (g/g)	Morphology
20	80	73	22	0,43	0,27	0,23	Heavy suspension
40	80	16	6	0,16	0,42	0,37	Heavy suspension
60	80	8	2	0,11	0	0,19	Heavy suspension
80	80	6	0	0	0	0,21	Heavy suspension
120	80	6	0	0	0	0,20	Heavy suspension
180	80	6	0	0	0	0,21	Heavy suspension

Anaerobic growth of *M.indicus* on synthetic medium with 30 g/l glucose, were carried out in 300 ml Erlenmeyer flasks. In order to evaluate ethanol tolerance of this fungus, after 13 hours of cultivation different amounts of ethanol (20, 40, 65, 85, 120 and 180 g/l ethanol) plus 50 g/l glucose were added to each flask.

Anaerobic cultivation with 20 and 40 g/l addition of ethanol increased the lag phase for about 10 hours before exponential growth began. Addition of ethanol also caused a high reduction rate of glucose consumption. In flask with addition of 20 g/l ethanol, after 150 hours of cultivation the strain was able to utilize only 70 g/l glucose. The anaerobic cultivation of extra addition of 40 g/l ethanol decreased the consumption rate of glucose even more greatly. Only 11 g of glucose were consumed after 90 hours of cultivation. The most important results are summarized in figures 14 -15 and tables 12.

During the cultivation, the most important by-product was glycerol and only one type of morphology, heavy suspension, was observed during the cultivation.

Only 1-2 g glucose was consumed by these fungi in the medium with extra addition of 60 g/l ethanol, after 175 hours of cultivation. Higher addition of ethanol almost no growth occurred during the cultivation.

3.4. Different sugar consumptions by *Mucor indicus* in anaerobic cultivation

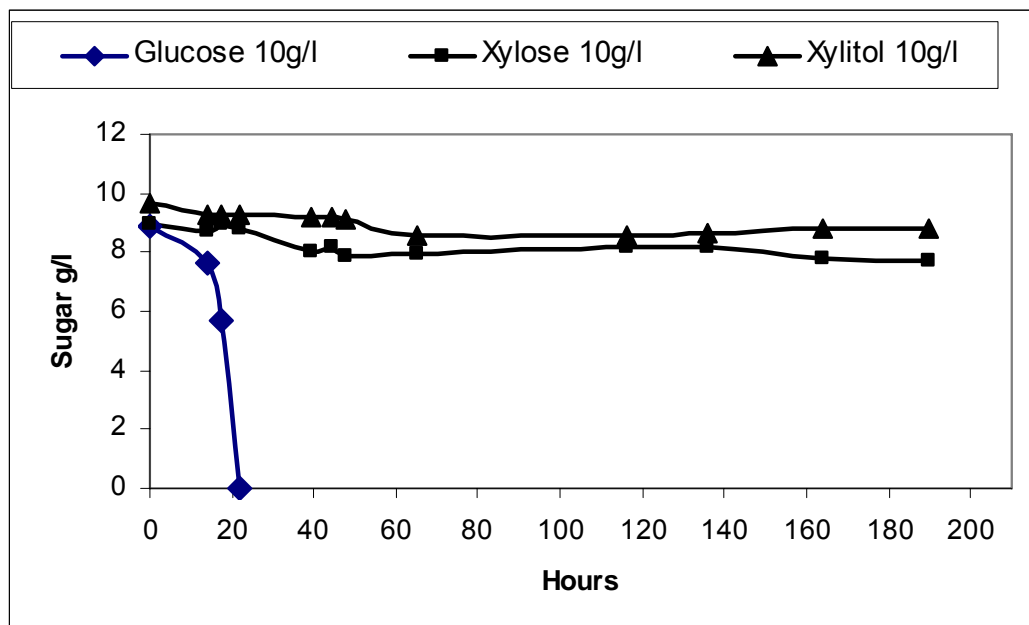


Figure 16. The capability of *M.indicus* CCUG22424 to consume the sugars.

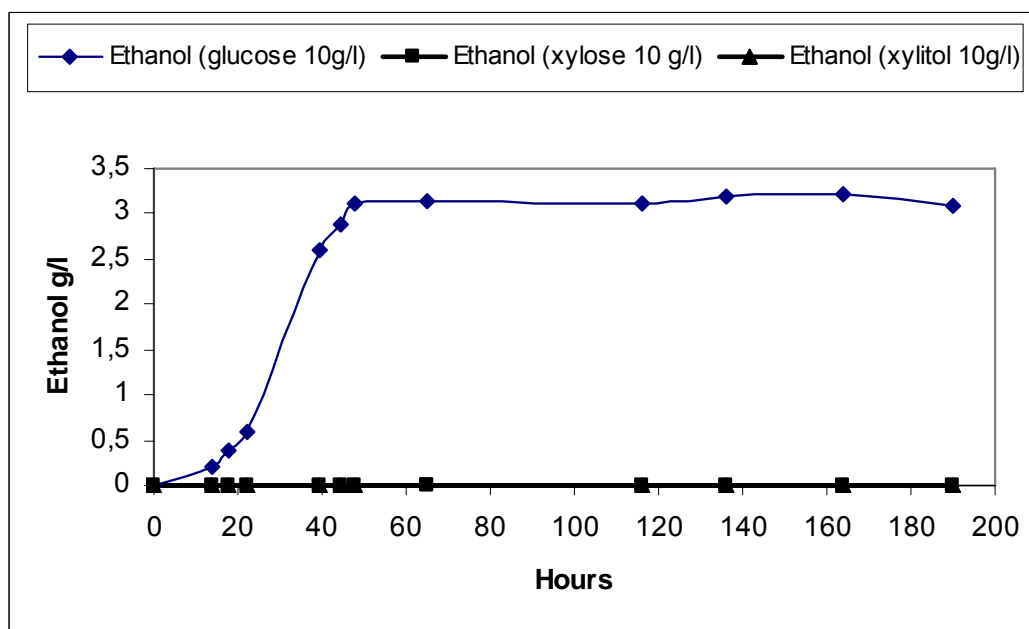


Figure 17. The capability of *M.indicus* CCUG22424 to produce ethanol

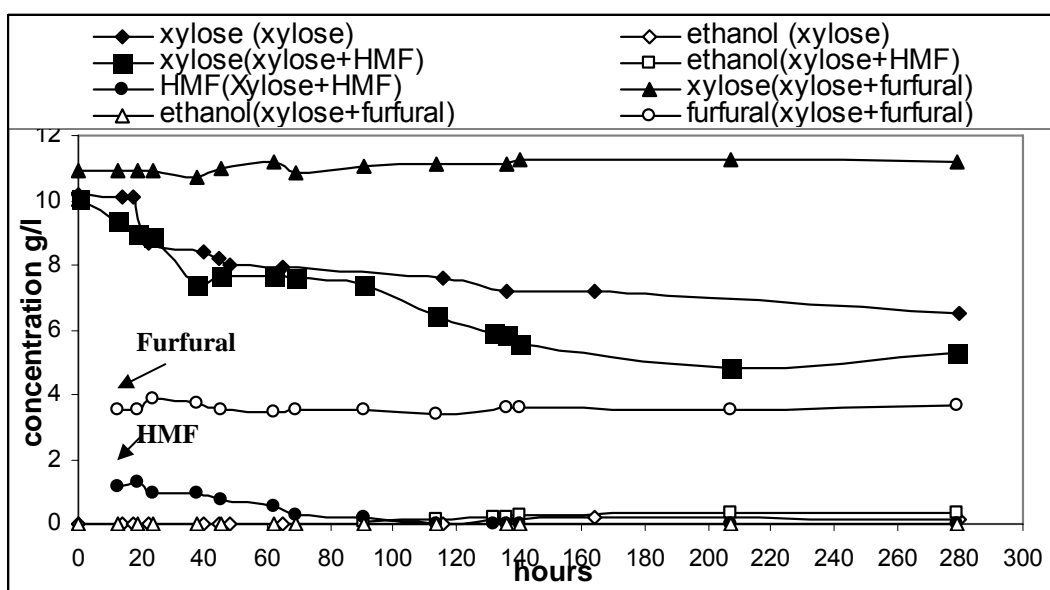


Figure 18. The capability of *M.indicus* CCUG22424 to consume the sugars and produce ethanol.

Table 13. Total sugar consumption, max. ethanol production and ethanol yield of *M.indicus* CCUG2242 in an anaerobic condition on a synthetic medium.

Sugar 10 g/l	Sugar consumption (g/l)	Max. ethanol production (g/l)	Yield ethanol
Glucose	10	3	0,32
xylose	2	0	0
Xylitol	≈ 1	0	0
Xylose + HMF	4,5	0,3	0,07
Xylose +Furfural	0	0	0

The capability of this fungus to consume different kinds of sugars was investigated by cultivating this strain in shake flasks containing either glucose, xylose and xylitol (10 g/l) as carbon and energy source in anaerobic condition for 190 hours. The most important results are summarized in figure 16 -18 and table 13.

M.indicus CCUG22424 consumed all glucose and produced ethanol while xylose and xylitol remained in the medium and there was no production of ethanol, even after 190 hours of cultivation. In order to increase the consumption of xylose; HMF and furfural were added as electoreceptors, after 12 hours of cultivation. The ability of xylose consumption and ethanol production of this fungus increased about 12% in the presence of HMF. The addition of furfural did not affect the consumption of xylose by this fungus.

The initial concentration of 1,2 g/l HMF was completely consumed while the furfural 3,5 g/l was not consumed at all.

4. Discussion

The most commonly used microorganism for industrial ethanol production is *S.cerevisiae* but the related previous research showed that *M.indicus* can be a good alternative to *S.cerevisiae*. In respect to ethanol yield and ethanol productivity *M.indicus* is similar to *S.cerevisiae*. The fungus was able to consume xylose and produce ethanol in the aerobic cultivation, which is a major advantage over *S.cerevisiae* [33], and also the cell walls of this fungus contain a great amount of chitosan, which has many applications [39-40].

The purpose of this study was to find out the glucose tolerance of *M.indicus* CCUG22424 in aerobic and anaerobic conditions and the ethanol tolerance of this fungus in anaerobic condition. In addition to that, sugars consumption and ethanol production of *M.indicus* CCUG22424 on glucose, xylose and xylitol were also investigated. In order to study the consumption of xylose a little further, furfural or HMF were added as an electro-acceptor to the medium which contained xylose as the carbon and energy source for anaerobic cultivation.

However, the results show that the glucose tolerance of this fungus in aerobic and anaerobic cultivation is almost the same. It is between 190 to 200 g/l glucose. The glucose tolerance of *M.indicus* CCUG22424 is less than glucose tolerance of *S.cerevisiae*. The results show that the ethanol tolerance of *M.indicus* CCUG22424 under anaerobic condition is around 70 g/l, which is also much lower than *S.cerevisiae* ethanol tolerance. *S.cerevisiae* can tolerate ethanol concentration as high as ca. 20% of fermentation medium [46].

The glucose concentration in the medium had a great impact on the lag phase, glucose consumption and ethanol production in both aerobic and anaerobic conditions. The lag phase increased as the initial concentration of glucose increased. While the glucose concentration increased above 190 g/l in the medium the glucose consumption and ethanol production decreased in both aerobic and anaerobic conditions. The reason for this could be that while the glucose or ethanol concentration in the medium increase the glucose and ethanol acts as inhibiting compounds and prevents the cells growth which leads to less glucose consumption and less ethanol production .

In aerobic condition, the morphology of this fungus depends on the concentration of glucose in the medium. In low concentrations of glucose the morphology is cotton-like but in higher concentrations of glucose the morphology is heavy suspension. In the anaerobic condition the morphology of this fungus is independent on the concentration of glucose (up to 400 g/l glucose).

Under anaerobic condition *M.indicus* is not able to utilize xylose but with the present of HMF, as electroreceptor, the consumption of xylose and production of ethanol increased, but it is still very low. The addition of furfural did not affect the fermentability of *M.indicus* CCUG22424.

5. Conclusions

- The glucose tolerance of *M.indicus* is around 190 g/l in both aerobic and anaerobic condition
- The ethanol tolerance of *M.indicus* is around 70 g/l ethanol in anaerobic condition
- In aerobic condition the morphology depends on the concentration of glucose in the medium, in low concentrations the morphology is cotton-like but in higher concentrations of glucose the morphology is heavy suspension
- Under anaerobic condition *M.indicus* is not able to utilize xylose but with the present of HMF, as electroreceptor, the consumption of xylose and production of ethanol will increased but it is still low

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