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Enzymatic Hydrolysis Of Cellulose: Isolation And Investigation Of Cellulose Breaking Fungus From Soil

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Abstract:

Enzymatic hydrolysis of cellulose is an important reaction in nature and marks the first step in the decay of cellulose, the most abundantly occurring organic material. Unlike the chemical reactions, the enzymatic process has not yet been adapted to any useful industrial purpose. Cellulosic biomass is very difficult to degrade because of its recalcitrance. Today it is mostly pretreated with thermo chemical methods to make it more accessible to enzymes, which are produced in an extra step and then added to the biomass. This technical paper presents series of experiments done in isolating cellulase (a cellulose breaking enzyme) producing fungus from paper samples kept in natural conditions at various locations. The enzyme producing capabilities of various samples, the optimal conditions for fungal growth etc are herein presented in the paper.

1.Lignocellulosic Biomass

Cellulose is the most abundant polymer in the world. Lignocellulosic biomass makes about 50% of the total biomass in the world with an estimated annual production of 10-50 billion tons (Sanchez and Cardona 2008). It is the main component of plant cell walls, but it is also produced by some animals and bacteria. Cellulose makes a large fraction of the plant dry weight, being typically in the range of 35-50% by weight. It gives stability to the plants even in the absence of water and makes them more resistant. The basic structure of cellulose are 1,4-β-glycosidic linked D-glucose molecules that form unbranched chains, consisting of several thousand glucose molecules. These chains are packed to microfibrils which are held together by strong hydrogen bonds. The microfibrils are then assembled to cellulose fibers that are kept together by van-der-Waals forces and hydrophobic interactions (Himmel, Ding et al. 2007). The result of this process is a crystalline structure which makes it very difficult to degrade. The microfibrils are packed so tightly that enzymes and even water can hardly penetrate them. But the cellulose fibers are not completely crystalline; they contain several types of irregularities, such as micropores or kinks, with amorphous character. These regions are the points of attack for the cellulolytic enzymes. When the cellulolytic enzymes cleave the glucose chains, the results are so called cellodextrins. These are short glucose chains of various lengths. The shortest ones, i.e. glucose dimers, are called cellobiose and are sometimes not included in the cellodextrin classification. Because adjacent glucose molecules are rotated by 180° in cellulose, cellobiose is the shortest repeating unit in cellulose molecules (Lynd, Weimer et al. 2002).

Furthermore, the cellulose fibers are embedded in a matrix of other structural polymers such as hemicellulose and lignin. Hemicellulose is a mixture of short linear and branched polymers consisting of different pentose and hexose sugars. 20-35% of the plant dry weight consists of hemicellulose, thus it is the second most abundant polymer in the world. It mostly consists of xylose, a pentose sugar, which implies a problem for Bioethanol production, because not all microbes can metabolize xylose. If it is not possible to overcome this problem, a large fraction of the sugars in the cellulosic biomass will not be available for fermentation. Lignin is a complex macromolecule that is made of three different types of phenolic monomers. These three monomers - p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol - are the basis to form a variety of cross linking motifs. The structure of lignin makes it the most recalcitrant substance of the three and currently there are no processing methods that make it available to

fermentation. However, as its energy content is high, it can be separated from cellulose and hemicellulose and then be burned to produce electricity, but it can also be used to produce other chemicals from its constituents (Chang 2007).

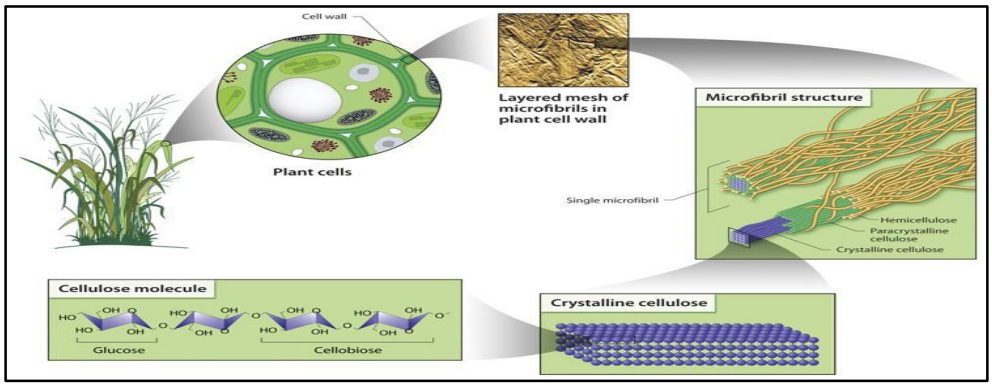

Figure 1: Structure and composition of cellulose fibers. The glucose chains are held together by hydrogen bonds and form crystalline cellulose that is arranged in microfibrils. These are linked by hemicellulose and lignin, forming the recalcitrant cellulose fibers. (DOE 2005).

2.Processing Of Ligno-Cellulosic Mass

Today's processing of cellulosic biomass to produce biofuels can be divided into different steps: first, the feedstock has to be harvested; besides the choice of the feedstock and the growing and harvesting techniques, this step also includes the engineering of the plants themselves, to make them produce biomass that is easier to process. As this is beyond the scope of this paper, it will not be covered. Second, the biomass is pretreated thermo-chemically to remove lignin, solubilize hemicellulose and make the cellulose more accessible. The third step is the enzymatic hydrolysis that converts the cellulose to sugar. The enzymes that are needed for this step are usually produced in an extra step and then added to the biomass. Fourth, the sugars are fermented to ethanol by microorganisms and the ethanol is removed from the bioreactor. Additionally, the ethanol is usually distilled after the extraction, because the ethanol needs to be highly concentrated in order to be used as fuel additive. In order to get an efficient process, some of the waste material can be reutilized in the process. Lignin for example can be burnt to generate electricity and heat for the process, but there are also technologies under development that would allow converting it to higher-value products. To minimize the fresh water consumption it can be reutilized, but the problem is that dissolved inhibitory substances might accumulate and interfere with the process. So the positive effects of water reuse and the negative effects of increased energy

and material use due to additional detoxification steps have to be balanced (Hahn-Hagerdal, Galbe et al. 2006).

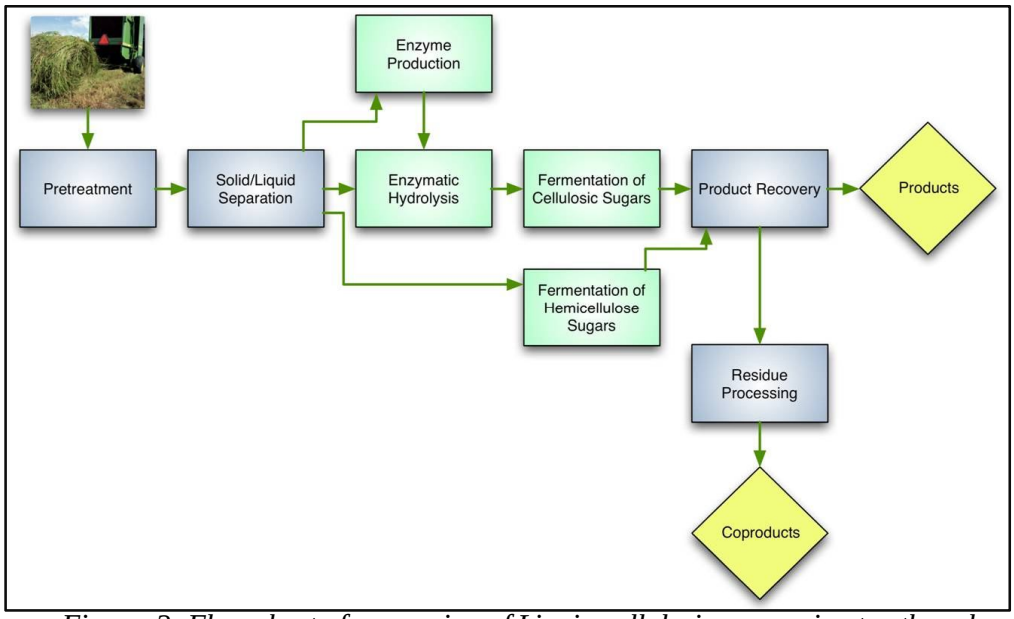

Figure 2: Flow chart of processing of Lignin-cellulosic conversion to ethanol (Picture from Michigan state University tech paper)

3.Pretreatment

The pure physical pretreatment methods consist mainly of chipping, milling or grinding the material to enlarge the surface area and remove the protection of epidermal tissue of the plant, such as the cuticle and epicuticular waxes. Pyrolysis, i.e. heating the material above 300 °C and then cooling and condensing it, has also been tested, because cellulose rapidly degrades at high temperatures. To which extent the material is treated depends on the material itself, (e.g. wood needs more treatment than straw) and on the benefits that can be gained by further treatment compared to the energy costs of the process. The physical pretreatment methods are rarely used alone, because of their high energy and capital costs (Sanchez and Cardona 2008).

Thermochemical pretreatment methods are thought to be more effective than pure physical methods and they are the furthest developed methods. There are a lot of thermochemical pretreatment methods currently available. Some of them are more promising than the others, but in the end the variability of the biomass in hemicellulose and lignin content makes it impossible to say which one is the best. The question is rather about finding the appropriate method for a given feedstock. The methods differ in their efficiency of xylose oligomers recovery, in the formation of unwanted byproducts

and in their consumption of energy and chemicals. But the aim of all of them is to increase the surface area or porosity to make the cellulose more accessible to enzymes, to solubilize hemicellulose and to solubilize or redistribute lignin. Redistribution of lignin is the melting of lignin and reconnection of it during the cooling to a new structure with modified properties that make the biomass easier to degrade. The most common pretreatment processes are: Dilute acid treatment, steam explosion at high solids concentrations, hydrothermal processes, organic solvents with water, ammonia fiber explosion (AFEX) and strong alkali processes using lime or NaOH as base.

4.Enzymatic Hydrolysis

To make the sugar monomers available for fermentation the cellulose and hemicellulose chains have to be further hydrolyzed after the pretreatment. There are a lot of microorganisms that produce a variety of enzymes that are able to do that, known as the cellulase systems. There exist both anaerobic and aerobic bacteria and fungi that produce cellulase systems. Anaerobic bacteria occur for example in the soil, in decaying plant material, in sewage plants and in ruminant animals and in the guts of termites, where they live in symbiosis with their hosts and process plant material for them. Common anaerobic cellulolytic bacteria are various Clostridium species. Aerobic bacteria are found in various habitats e.g. in water, in the soil, on decaying plant material or in animal feces. Cellulomonas and Streptomyces are two frequent genera of cellulolytic aerobic bacteria. Aerobic fungi play an important role in the degradation of plant material and are found nearly throughout the nature. A well known aerobic cellulolytic fungus is Aspergillus niger. Anaerobic cellulolytic fungi are less common and there exist only six recognized genera of them: Anaeromyces, Caecomyces, Cyllamcyces, Neocallimastix, Orpinomyces and Piromyces. They live in the intestinal tract of large herbivorous animals, like elephants, horses, cows or sheep (Doi 2008).

The cellulolytic enzymes can be divided into three groups based on their enzymatic activities: 1) endoglucanases 2) exoglucanases and 3) β-glucosidases. They all have in common the ability of hydrolyzing the 1,4-β-glycosidic bond between the D-glucose molecules, but they differ in their starting point and substrate when hydrolyzing. Endoglucanases, also known as 1,4-β-D-glucan-4-glucanohydrolases, attach to the cellulose at arbitrary internal amorphous sites and cleave the polysaccharide chain by inserting a water molecule in the 1,4-β bond. The results are oligosaccharides of various

lengths with a reducing and a nonreducing end. The exoglucanases start at either the reducing or nonreducing end of these oligosaccharide chains and release either directly glucose or the cellobiose dimer. The glucose releasing enzymes are called glucanases and the cellobiose releasing enzymes are cellobiohydrolases. The exoglucanases can also work autonomous and peel cellulose chains from microcrystalline cellulose. Finally the β-glucosidases (or β-glucoside glucohydrolases) hydrolyze the cellobiose dimers and the cellodextrins of various lengths to glucose (Lynd, Weimer et al. 2002; Himmel, Ding et al. 2007).

5.Fermentation

After the hydrolysis the sugars have to be fermented to ethanol. The hydrolyzate now contains various hexoses, mainly glucose, and pentoses, mainly xylose, and depending on the substrate and the pretreatment method various byproducts. Currently the fermentation is mostly done with S. cerevisiae (commonly known as baker's yeast) because of its well known characteristics, robustness and high ethanol yield. However, S. cerevisiae can only ferment hexoses and not the pentoses. The pentoses can be fermented in an additional step by another organism, or S. cerevisiae can be genetically engineered so that it is able to ferment pentoses as well (van Zyl, Lynd et al. 2007). Besides a high yield, an important issue with fermentation is the tolerance of the fermenting organisms to the product itself. A strategy to overcome this is to have a system where the ethanol is removed continuously to keep the concentrations low. Another problem is inhibitory compounds that are produced during the thermochemical pretreatment. As mentioned above they can be reduced by an additional detoxification step, but this is expensive. Another way would again be to improve the tolerance of S. cerevisiae towards inhibitors (van Maris, Abbott et al. 2006).

6.Experiment Aim and Scope

Investigating samples collected from various regions for microbes which possess the capability of producing enzymes was the real motto of the experiment. Moreover experiments were also performed to determine its cellulolytic potential and optimal growth conditions.

6.1.Sample collection

Normal printing paper drenched in water was placed one inch within the soil at 4 various locations. After a week this paper samples were collected and checked for degradation of paper. The paper was checked for firmness and color change. One of the sample showing maximum degradation was selected for further investigation.

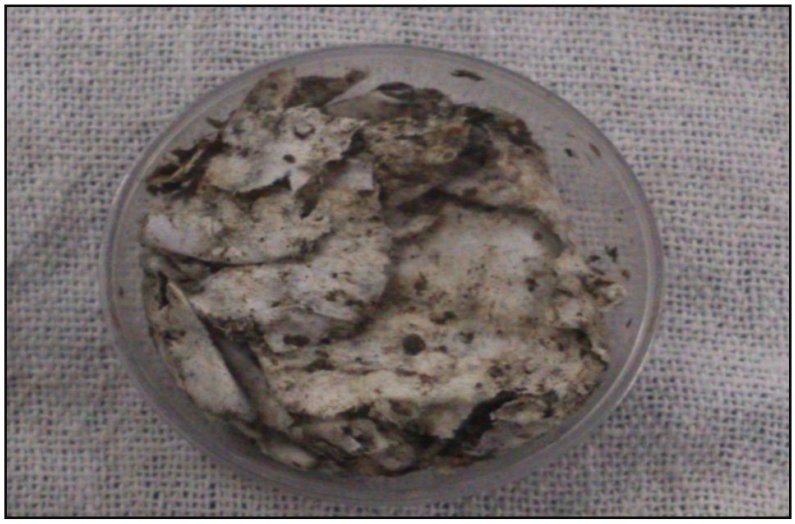

Figure 3: The paper sample after being dug in the soil under natural conditions for seven days.

7.Isolation And Screening Of Cellulose Breaking Fungus

The paper was carefully checked for any vital regions which shows cellulose breakdown. Such regions were torn and then inoculated in a basal salt media ($NaNO_3$ -2.5 g; KH_2PO_4 - 2 g; $MgSO_4$ 0.2 g; NaCl - 0.2 g; $CaCl_2 \cdot 6H_2O$ - 0.1 g in a liter) . The mixture is filtered using Whatman filter paper no. 1 for the isolation of cellulolytic fungus. These cultures were incubated for 7 days in a shaker incubator at 37°C at 100 rpm. Fungal colonies capable of utilizing cellulose as sole source of carbon were isolated on cellulose agar media composed of KH_2PO_4 -0.5 g $MgSO_4$ -0.25 g, cellulose - 2.0 g, agar -15 g, gelatin -2g and distilled water lL and at pH 6.8-7.2.Confirmation of cellulose-degrading ability of bacterial isolates was performed by streaking on the cellulose Congo-Red agar media with the following composition: KH_2PO_4 -0.5 g, $MgSO_4$ -0.25 g, cellulose -2 g, agar -15 g, Congo-Red -0.2 g, and gelatin -2 g; distilled water -1 L and at pH 6.8-7.2. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies, and only these were taken for further study. Cellulose-degrading potential of the positive

isolates was also qualitatively estimated by calculating hydrolysis capacity (HC), that is, the ratio of colony of clearing zone and colony.

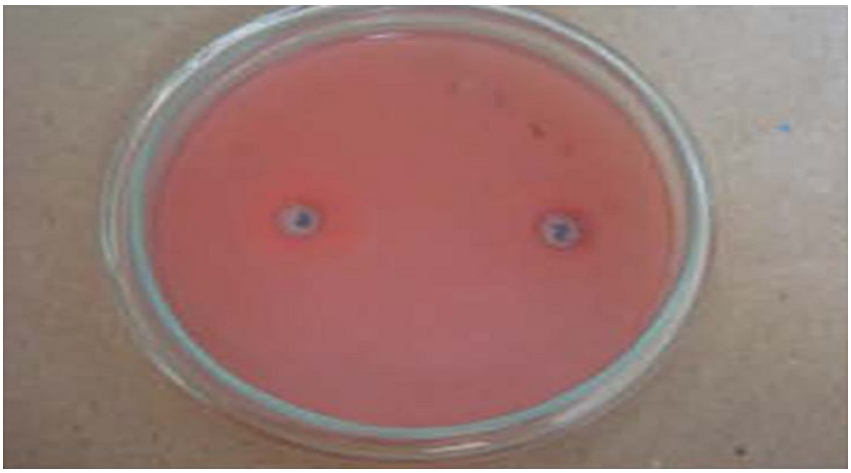

Figure 4: Formation of clear zone to indicate cellulase formation

8.Fungal identification

The fungus isolated from the above technique was presumptively identified by means of morphological examination and comparison with images from Wikipedia.

9.Conclusion: Strain Of Fungus Trichoderma

9.1.Process For Optimization

The factors like temperature, pH, and different substrate affecting the production of cellulase production were optimized by conducting the experiment at various conditions. The experiment was conducted in 250 mL conical flask. After sterilization by autoclaving the flask was cooled and inoculated with cultures at 30 °c for 72 hours under various experimental conditions as described below. The fermentation was carried out at various temperatures ranging from 25°c to 45°c to study their effect on cellulase production. The pH was varied from 3 to 11 with 1N HCl and 1N NaOH. Different substrate were chosen like corn cobs, rice bran, wheat bran, cellulose and CMC (Carboxy methyl cellulose). The supernatant was collected, centrifuged at 1000 rpm for 15 minutes and the liquid was used for further tests.

9.2.Estimation Of Enzyme Activity By Apple Juice Clarification Test

Cellulase is one of the most common enzymes used for clarifying fruit juices. Apple cell wall contains pectin which is removed by cellulase. The test is as mentioned under

- Cloudy pure apple juice (5mL) and cellulase enzyme (1mL) obtained from the above process are stirred well in a test-tube to facilitate complete mixing of both the liquids.
- The test-tube is then maintained at 50°c by dipping it in a water bath whose temperature is regulated.

Figure 5: Clarification of apple juice using cellulase enzyme

10.Results Of The Experiments

The enzyme activity was measured as the height of the clarified apple juice in the test-tube.

1. Average Enzymatic action measured against time.

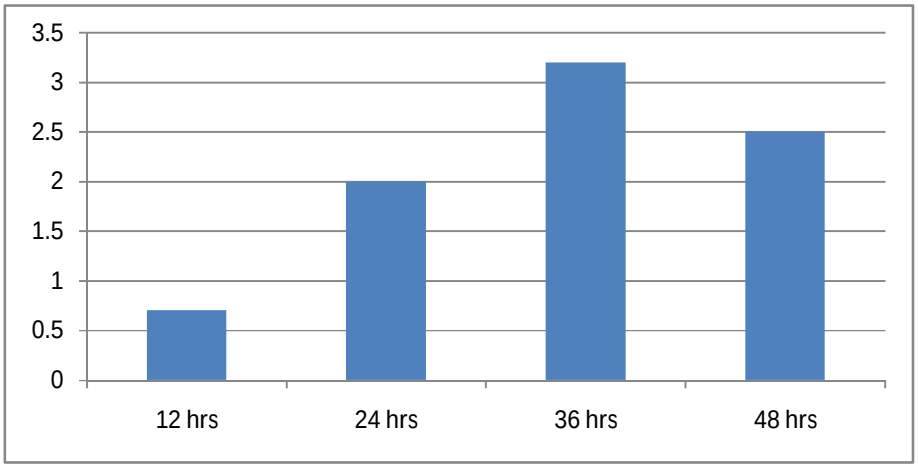

Figure 6

Average enzymatic activity in terms of height of clarified juice v/s time

Conclusion: Optimum enzyme production observed after 36 hours of fermentation.

2. Enzymatic action v/s pH at which fermentation takes place at constant temperature of 35° c and substrate corn cobs after 36 hours.

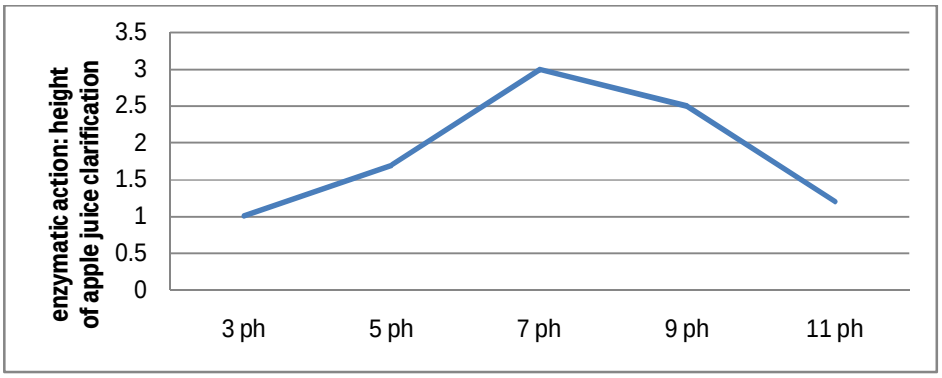

Figure 7

Conclusion: Optimum fermentation and maximum yield of enzyme formation takes place at pH 7.

3. Enzymatic action v/s Temperature at which fermentation was carried out at 7 PH and corn cobs as substrate after 36 hours.

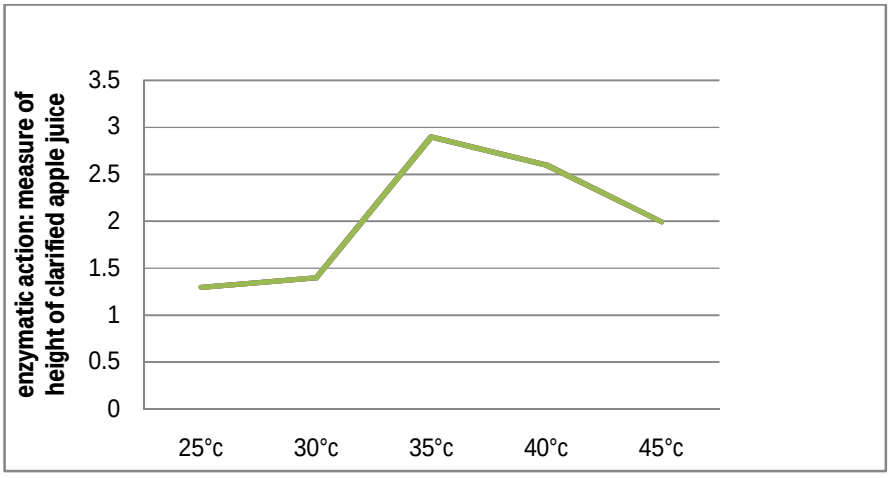

Figure 8

Conclusion: Optimum temperature for maximum enzyme production is 35°c.

4. Enzymatic production comparison with fermentation carried out with different substrate at 7 PH and temperature of 35°c after 36 hours.

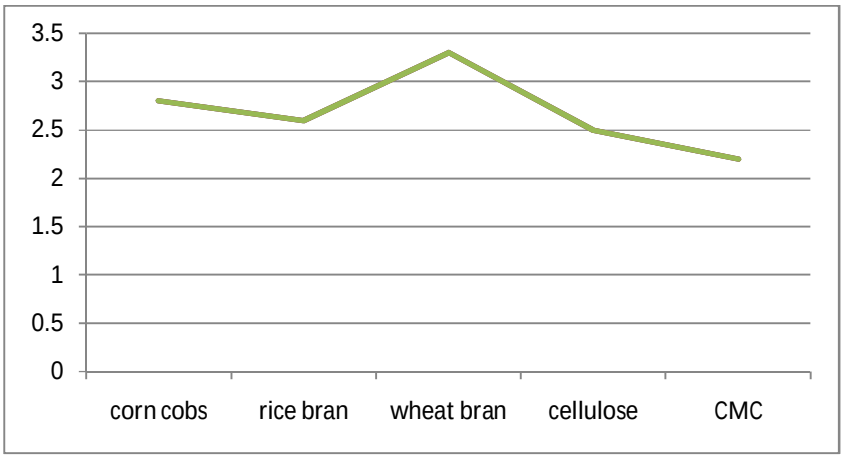

Figure 9

Conclusion: Wheat bran is the most suitable substrate for Trichoderma strain for enzyme production.

11.Conclusion For The Experiments

Optimum temperature: 35°c

Optimum PH: 7

Time taken for optimum yield of enzyme: 36 hours

Best substrate material: Wheat bran

12.Final Conclusion

Ethanol from lignocellulosic biomass has a great potential as an environmentally sustainable alternative to fuels that are derived from crude oil. Today, however, there is no processing technique that produces ethanol at costs comparable to gasoline, but as oil prices are rising and new technologies are emerging, this might change. A milestone in the scientific development would be microorganisms that can convert lignocellulosic biomass into ethanol efficiently and therefore would enable consolidated bioprocessing to become commercially attractive. There are naturally and recombinant cellulolytic organisms that are capable to do that but the achieved yields are low and they lack the robustness that is needed in an industrial process .

Today there seems to be more work ongoing in the field of genetic engineering of noncellulolytic microorganisms. Despite the large efforts that have been made, there are still no recombinant microorganisms that achieve high ethanol yields. From our point of view the use of more robust funguses found in the nature should be competent in faster

enzyme production than trying to introduce a very complex enzyme system into another organism. The recent success from Shaw, Podkaminer et al. (2008) seems to confirm this assessment. Additionally the native cellulolytic strategy is appropriate for evolutionary engineering which might be an advantage when implementing the processes on an industrial scale as there are still concerns about the use of genetically engineered organisms, especially in Europe. In the end one can just speculate why the native cellulolytic strategy has been neglected since the 1980's.

Furthermore it is very important to keep in mind that not only scientific, but also sociopolitical and environmental aspects have to be considered. It is not sufficient to develop efficient and affordable processing techniques to get sustainable fuels. Also the production of the feedstock must be sustainable and should not compete with other more basic requirements of society. Especially in poor countries that might see biofuels as a chance to make a lot of money the incentive to clear all their forests might arise. The developed countries with their demand of renewable energy sources therefore also have to take responsibility. However, ethanol that is produced from lignocellulosic biomass can solve the current conflict between food and fuel production that emerged with the first generation biofuels and can make a contribution to renewable energy production.

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