Integrated Storage and Pretreatment of Wheat Straw for Biofuel Production

Harikrishnan Anjaly Sukumaran Nair

Uppsala BioCenter
Department of Microbiology
Faculty of Natural Resources and Agriculture Sciences
Swedish University of Agricultural Sciences
Uppsala

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Supervisor: Matilda Olstorpe
Examiner: Volkmar Passoth

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ABSTRACT

The study aims for the development of a Pretreatment method for wheat straw, when it is stored at low temperature. The hypothesis is that an initial disruption of the crystalline nature of lignocellulose can improve the efficiency of current pretreatment method during low temperature storage. A silo model storage of wheat straw, with high moisture, involving 3 different fungal species (*Holtermanniella takashimae , Pichia anomala* and *Anthracophyllum discolor*) and their combination at two different temperatures (4°C and 15°C) was tested. The microbiology of samples was studied for analyzing the effectiveness of conservation. Dilute acid treatment was done prior to the saccharification with Accellerase™ 1000 enzyme. The soluble fraction of hydrolysate was fermented in 15 ml serum flask with *Saccharomyces cerevisiae*.

The biomass stored with combination of *Holtermanniella takashimae and Pichia anomala* at 4°C showed significant improvement in initial ethanol yield (2.8% increase in ethanol at P-value<0.05) compared to the non inoculated wet control sample. A detailed study of the simple sugars released, showed that the total sugar yield for *P. anomala* inoculated sample was double as that of control sample (29.283g/L Vs 17.43g/L). The ethanol yield for *A. discolor* inoculated samples (59%) was higher than the theoretical maximum (<50%), which suggests that the saccharification may not have completed at the time of fermentation.

The samples stored with *P. anomala* showed significant inhibition of mold and other contaminants. But the results were compared with wet non inoculated samples which were non sterile and had high microbial load (10^6 CFU/g of fungus, 10^8 CFU/g aerobic bacteria, 10^7 CFU/g of enterobacteria) during incubation. So it is suggested to carry out a future study with simultaneous saccharification and fermentation (SSF) to completely utilize the free sugars and with a dry stored material as control, to avoid the effect of natural microflora.

POPULAR ABSTRACT

The ever growing human population and shrinking arable land areas cause the first generation biofuels derived from edible biomass, such as corn and sugar cane made as a non feasible option. In the 21st century, we need to develop a new way to fuel our cars without competing with the food and reduced environmental impact. The second generation biofuel, so called cellulosic ethanol can be produced from literally any part of biomass, and can be one contribution for our growing fuel needs. But the technologies for converting the plant biomass into fuel are not yet a reality. Primarily, the conversions of cellulose to fermentable sugars (the pretreatment of material) still acts as a bottle neck in the process. Storage of biomass is an important issue of biofuel production since we need a constant supply of material for industrial production processes. In Sweden, the normal conservation of material by drying should be replaced by wet silo model of storage. In the current project, we have developed an ingenious solution for breaking down the hard cellulosic structure during the time of storage itself in the presence of high moisture, making it more accessible to the further enzymatic steps without increasing the cost of production. We have used 3 fungal species which can grow in low temperature, and still disrupt the complex cellulosic structure, and studied the effect for ethanol yield. Storing the wheat straw at higher moisture level with our combination of fungal species was able to increase the ethanol yield by approximately 2.8%. But there were some more aspects of this study, which need to be extended since the experimental design was unable to include the insoluble part of the hydrolysate into ethanol. So a future study with the proposed changes may be a breakthrough in the cellulosic bioethanol industry, making it as a reality.

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1. INTRODUCTION

It is important to have a liquid fuel replacement for petroleum, as the world's fossil fuel supply is limited. Ethanol is one of the most attractive biofuels in the 21st century mainly because of its renewable nature, and lower environmental impact than the conventional fossil fuels. But currently produced ethanol, mainly form corn (starch) and sugarcane is not sustainable as it can cause competition with food, and creates other major problems like land use issue, green house gas release[1] etc. The second generation biofuel[2] or lignocellulosic biofuel can be produced literally from any cellulosic materials like wood chips, agricultural residues and special energy crops, making it a global fuel[3]. But cellulosic biofuel is not yet a reality since the production of fermentable sugars from cellulose is not economically viable and current methods of pretreatment (Physical and chemical breakdown of the material to make it more accessible for the subsequent hydrolysis step) are quite harsh, resulting in the production of highly toxic fermentation inhibitors like furfural and hydroxymethyl furfural [4].

The common fermentation yeast *Saccharomyces cerevisiae* can only ferment hexoses and few disaccharides [5], whereas the plant material usually contains practically no glucose, but in abundance of polysaccharides cellulose and hemicellulose entrapped in phenolic lignin compounds. The direct conversion of lignocellulose to glucose is practically impossible, because of the rigid and polymer nature of wood. Currently an additional step called thermo chemical pretreatment, is used to breakdown the polymer nature of plant material making it more vulnerable for the further hydrolysis and saccharification process. But there are several disadvantages of this process. The harsh and costly pretreatment makes the pretreated hydrolysate rich in fermentation inhibitors and makes the process less feasible. Researchers around the world have tested several possible alternatives to the pretreatments but no substantial progress has been achieved. Hattakka in 1983 has proposed a biological pretreatment[6], where he used several lignolytic fungi to breakdown wheat straw and produce glucose in a sterile environment. Even though Hattakka succeeded to produce high amounts of glucose, without any inhibitors, the sterilization requirement and long incubation time (15days) made the pretreatment non feasible [7]. The combination of biological pretreatment with chemical treatment also failed since the high amount of sugar produced during biological treatment remains as a source of fermentation inhibitors in the further chemical steps. So it is clear that, the focus of this research is not to produce free sugar, but to disrupt the structure of lignocellulosic feed stock, without any further requirement of sterilization, material handling problems and cost. Working with non sterile feedstock creates several other problems such as difficulty in overall microbial control, lower yield and other hygiene related problems.

1.1. Energy efficient Storage

The primary target of cellulosic ethanol production is the net energy output. That is, achieving good bioethanol production with low energy and chemical input. Currently 32% of total cost investment is for the pretreatment and chemicals [8].

The traditional way of conservation of biomass is based on drying the material which reduces the water content and in turn prevents the growth of spoilage microorganisms. Storage of feed stock will become more important when industrial production of biofuel needs constant flow of material, whereas the biomass production process is seasonal. But the currently practiced drying is energy intensive and much difficult in areas like Sweden, where the vegetation period is rather short. In 2009 Passoth et al demonstrated that storing moist wheat grains in airtight situation improves the bioethanol yield [9]. The concept of integrated storage and pretreatment of biomass is based on storing materials with high moisture content, favoring useful micro organisms which allows partial breakage of cellulosic structure and eases the release of sugar in the later production process.

1.2. Biopreservation

The concept of integrated storage and pretreatment in countries like Sweden, involve biopreservation with low temperature preferring microorganisms, which can grow at typical Swedish temperatures and at the same time help in breaking down complex lignocellulose. At the same time working with non sterile material is rather troublesome, since the overall microbial characteristic of the material depends on the initial microbial load also. So it is desired to have a microorganism which has the ability to control (inhibit the growth) other microorganisms. *Pichia anomala* is known as a potential biopreservative agent [10] and known to improve feed hygiene in moist wheat grains. Also the ability of *P. anomala* to grow in extreme conditions like low and high pH and low oxygen [11] makes it a good candidate for the current study.

Holtermanniella takashimae is a novel yeast species originally isolated from plant litter and agriculture land around the world [12]. *H. takashimae* is able to grow at low temperatures (4°C) and able to degrade cellulose. Co-cultivation of *H. takashimae* with *P. anomala* may result in a symbiotic relation where *H. takashimae* degrades cellulose and provides *P. anomala* with enough glucose to survive, which in turns kills all other contaminants including *H. takashimae*.

White rot fungi belong to the most efficient wood degrading microorganisms [13], widely known for their ability to produce extracellular enzymes like Lignin peroxidase (Lip), manganese peroxidase (MnP) and laccase [14]. *Anthracophyllum discolor* was shown to be able to grow in wheat straw and compete with other natural soil microorganisms in non sterile conditions and produce lignolytic enzymes[15].

2. AIM

The aim of this study was to establish an energy efficient integrated storage and pretreatment of wheat straw for ethanol production.

The study also aimed for microbial characterization of stored wheat straw at high moisture level, and to optimize a smaller scale pretreatment system for storage studies in a controlled environment.

3. MATERIALS AND METHODS

3.1. Wheat straw

Dry wheat straw used was obtained from SLU. It was then milled into fine powder in an Ultra centrifugal mill ZM 1000 (Retsch Germany). Milled materials were stored airtight in a plastic bag in 4°C until use. The original water activity (a_w) of the material was 0.233 corresponding to 5.72 % moisture.

3.2. Water activity

Water activity (a_w) is a measurement of free water content. It is defined as the partial vapor pressure of water in the substance divided by that of pure water at the same temperature [16]. Higher a_w substances tend to support more microorganisms. Bacteria usually require at least a_w 0.91, and fungi 0.7. If a product is kept below a certain water activity, then mold growth is inhibited. This results in a longer shelf-life. Water activity was measured in Aqua Lab CX-2, (Decagon Devices inc. Washington, USA). Moisture content was measured in Sartorious Moisture analyzer MA-45 (Göttingen, Germany).

3.3. Fungal strains

The Fungal strains used in the study are shown in the table (table: 1).

Table 1: Fungal strains used in the study. CBS stands for the culture collection in Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht Netherlands. J Represents the culture collection in the department of microbiology.

Yeast strain	Known Activity
Holtermanniella takashimae J596(CBS 10506)	Cellulolytic enzyme
Pichia anomala J121	Biopreservative yeast
Anthracophyllum discolor†	Lignolytic white rot fungi
Saccharomyces cerevisiae J672	Fermentation yeast
Pichia stipitis J563 (CBS 5774)	Xylose fermenting yeast
Dekkera bruxellensis J355 (CBS 74)	Alternative fermentation yeast

† Not deposited in any culture collections. Donated by Dr. Leticia Pizzul, Dept of Microbiology, SLU.

3.4. Media composition

3.4.1. *Yeast Extract- Peptone- Dextrose Medium (YPD)*

YPD medium (Yeast extract 10 g/L, Peptone 20 g/L from Oxoid LTD Basingstoke, New Hampshire, Glucose 20 g/L (Duchefa Biochemie B.V, Duchefa Netherlands) were used for yeast growth. 15 g/L Agar (Oxoid LTD Basingstoke, New Hampshire) was used for solid plates. 0.1 g/L of Chloramphenicol (Sigma Aldrich Chemie GmbH, Steinheim, Germany) was added for preventing bacterial growth.

3.4.2. *YNB media*

The Yeast Nitrogen Base 6.7 g/L (difcoTM, Becton Dickinson and Co, USA) was filter sterilized (0.2 µm) (Sarstedt, AG & Co, Nümbercht; Germany). Glucose

solution that was separately autoclaved was added to the YNB for a final glucose concentration of 20 g/L.

3.4.3. de Man, Rogosa and Sharpe (MRS) Media

MRS (62.2 g/L, Merck kGaA, Darmstadt, Germany) media was used for selective growth of Lactic acid bacteria. The commercial preparation of MRS was prepared according to the manufactures recommendation and supplemented with 0.09 g/L Delvocid ® (DSM Food Specialties, Delft, Netherlands) as fungal inhibitor.

3.4.4. Violet Red Bile Agar with Glucose (VRBG)

VRBG (38.5 g/L Merck kGaA, Darmstadt, Germany) contains violet red dye with bile salt. This promotes selective growth of bile-tolerant gram negative Enterobacteria. VRBG plate count is used as a measure of the hygienic state of the sample [17].

3.4.5. Tryptone Glucose Extract Agar (TGEA)

TGEA (24 g/L, Oxoid LTD Basingstoke, New Hampshire) was used for the enumeration of aerobic bacteria.

3.5. Inoculum preparation

All strains were grown on YPD master plates and stored in a 2°C incubator. For liquid culture *P. anomala* was incubated in YNB medium and incubated at 25°C for 24h at 140RPM. *H. takashimae* was incubated at 15°C for 48h to reach substantial cell quantity, since the growth rate was very low. The cells where quantified using Hemocytometer (Scherf, Burker, Germany), and inoculated at 10^5 CFU/g of wheat straw by diluting with Normal saline (0.9% Sodium Chloride Solution) or spinning down and resuspending the pellet in required amount of normal saline .

3.6. Experimental Setup

H. takashimae, P. anomala, and *A. discolor* were inoculated in pure culture. A combination of *H. takashimae* and *P. anomala* was also tested for the storage experiment. All materials were made into water activity of 0.93 a_W by adding distilled water in a mixer and allowed to stand for three days at 4°C in a plastic bag. To simulate the silo model storage 50 ml falcon tubes with wheat straw packed were used. The lids were closed loosely to simulate air leak. There were three parallels for each sample and tested in two different temperatures (15°C (A) and 4°C (B)). Three parallels of uninoculated wheat straw of 0.93 a_W were used as control. Another set of parallels was used for microbial quantification. Samples after the storage period were pretreated with dilute acid and proceeded for fermentation (Figure: 1).

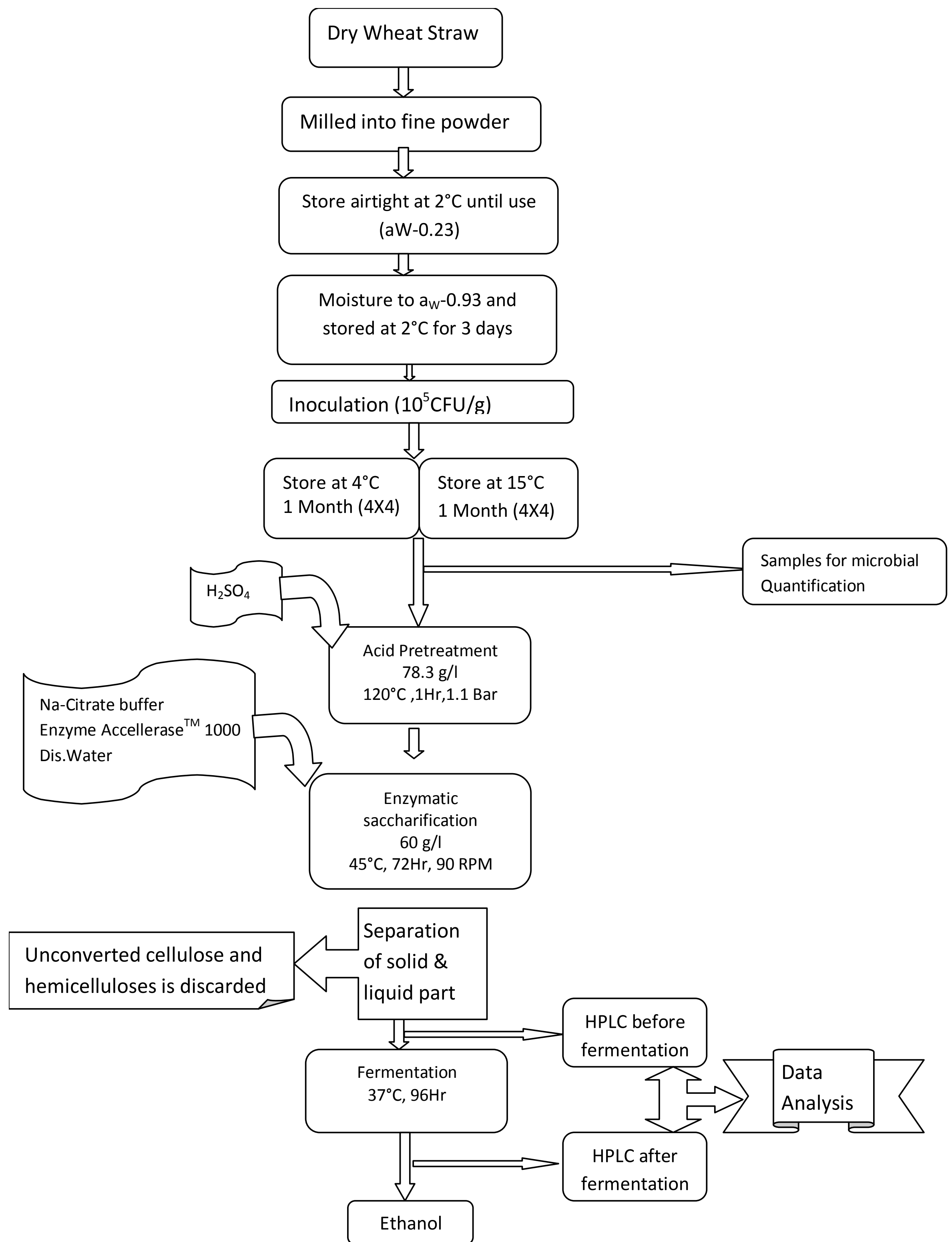


Figure 1: Flowchart of the experimental procedure

3.7. Phenol oxidase activity measurement

The assay is based on photometric measurement of color formation of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino)benzoic acid (DMAB) in presence of Hydrogen peroxide (H_2O_2) and Manganese sulfate ($MnSO_4$) [18]. 4 g of material was extracted with Succinate Buffer for 1 hour in a shaker [15], and the absorbance was measured in Kinetics Mode by Shimadzu UV-1800

UV-VIS spectrophotometer (Shimadzu Corporation Chiyoda-ku, Tokyo 101-8448, Japan) for the period of 3 minutes.

3.8. Dilute Acid Pretreatment

The wheat straw was converted to a slurry (78.3 g/L) by dilute Sulfuric acid (0.291 M)(Merk kGoA, Darmstadt, Germany) in 500ml conical flask and treated in an Uniclave 360 autoclave(Sjukhusservice AB, Vemdalem,Sweden), with constant time of heating (18-22 min) and cooling time approximately 35 min (120°C to 80°C). The bottles were allowed to stand for 1 hr at 121°C and 15 psi (1.1 Bar) pressure [19]. The pretreated sample was then adjusted to pH 5 by adding 250 g/L NaOH (Fig: 1).

3.9. Enzymatic saccharification

Accellerase^{TM} 1000 enzyme, which was a kind gift of Genencor Copenhagen, was used for the enzymatic degradation of pretreated materials to fermentable sugars. The enzyme preparation is a cocktail of several enzymes including β-glucosidase [20]**.** The saccharification was done at 6% dry matter of wheat straw. And the enzymes were added at the concentration of 0.1 g/g of dry mass (1g represents 2500 CMC U of Endoglucanase activity, 400pNPG of β-Glucosidase activity at 50°C and at pH 4.8). 1M Sodium Citrate buffer was used for keeping the pH optimum. The flasks were kept for shaking at 90rpm for 72hrs at 45°C.

3.10. Fermentation

Fermentation was conducted in 15 ml serum flasks with rubber stopper to obtain oxygen limitation along with 22 gauge needle for the CO_2 to escape. Water soluble sugar parts were filtered from the insoluble fraction using a 0.45µm filter. Yeast extract was added at the concentration of 5 g/L for providing the nutrient for yeast growth and ammonium sulphate ($(NH_4)_2SO_4$) (2 g/L) was added to provide ammonium salt [21]. Fermentation yeast *Saccharomyces cerevisiae* was added for final OD 1. Fermentation were done at 30°C for 4 days and the resulting media were analyzed for ethanol concentration by HPLC

3.11. HPLC analysis

The sugar and ethanol concentrations were analyzed by High performance liquid chromatography with refractive index detector in an Agilent 1100 series (Agilent Technologies;Waldbronn,Germany). The column used was HC-75 (Skandinaviska Genetec AB, Sweden) at 60°C with 0.05 M H_2SO_4 as eluent. Areas under the peaks were quantified by comparison with standard solutions of concentrations from 1 g/L to 30 g/L.

A Dionex (Sunnyvale, CA; USA ICS-3000) High-performance anion exchange coupled with pulsed amperometric detection (HPAE-PAD) was used for measuring mono- and disaccharides. The system consists of a 2 × 250 mm analytical CarboPac PA1 column at 30°C coupled with gold working electrode running in the integrated amperometry mode. A gradient method was used for the determination of maltose and cellobiose where 100 mM NaOH without (eluent A) and with 200 mM sodium acetate (eluent B) were used as eluents, with a gradient from 0 to 85 % B in 25 minutes at a flow rate of 0.25 ml/min. The standard curve for calibration was made with a mixture of maltose and cellobiose ranging from

10 to 100 µM in concentration. Other monosaccharides were separated by isocratic method using 15 mM NaOH at a flow rate of 0.25 ml/min[9].

3.12. Student T-test

A student's *t* test was performed to find out the significance of yield differences. A homogenous, 2 tail normal distribution was done at a significance level of P-value <0.05

4. RESULTS AND DISCUSSION

4.1. Moisture analysis

The dry weight of original wheat straw was 94.28 %(0.225a_w) corresponding to 5.72 % moisture. It is impossible to grow yeast on this low moisture value[16], and it needs to be raised to allow the yeast to grow. In order to find the optimum moisture content for the yeast cell to grow, a series of water activity tests were performed, by inoculating *P. anomala* in different tubes with increasing moisture level.

4.2. Water activity test

Tubes containing wheat straw of different water activity were inoculated with yeast samples to reach a cell concentration of 10^4 CFU/g of wheat straw and incubated at 15°C for 2 week (Table: 2). The colony count of yeasts at water activity 0.93 was considered as optimum and further experiments were done by adjusting the a_w to 0.93

Table 2: Fungal growth in different water activity mini silos. E0-E6 represents Tubes 50ml Falcon tubes filled with wheat straw of increasing water activity)

Sample	Water activity	Fungal colony
E0	0.225	**$2X10^2$**
E1	0.748	**$21X10^4$**
E2	0.840	**$23X10^5$**
E3	0.853	**$112X10^5$**
E4	0.928	**$47X10^7$**
E5	0.947	**$21X10^7$**
E6	0.966	**$123X10^5$**

4.3. Microbial characteristics

4.3.1. Natural micro flora of the sample

The wheat straw may already contain a consortium of microorganisms. Since the water activity was kept low by drying, the microbial growth was suppressed in the original sample, even though there could be some dormant activities, which could proliferate on suitable conditions. The control in our experiment (E0) was high moisture level 0.93 a_w non inoculated sample. So the microbial flora of mini silo-E0 was assumed as the natural micro flora in the experiment.

4.3.2. At the start of the experiment (t0)

The microbial load just after the addition of inoculum (Fig: 2) shows greater variation among fungal, Lactic acid bacteria and aerobic bacterial CFU. The samples should differ only by the amount of yeasts inoculum addition, and the effect of these yeasts on the growth of other microorganisms has to be

disregarded since the sample was standing only for less than an hour before it got plated, which is less than the doubling time of baker's yeast at 30°C. Also it is interesting to note that the uninoculated wheat straw also had 10^6 fungal CFU/g. That is the fungal populations in the inoculated samples may be a consortium of different fungal species along with the inoculated yeast species. Also the enterobacteria, indicating the hygienic state of sample had quite high initial load (~10^4 CFU/g of wheat straw) in the sample [22].

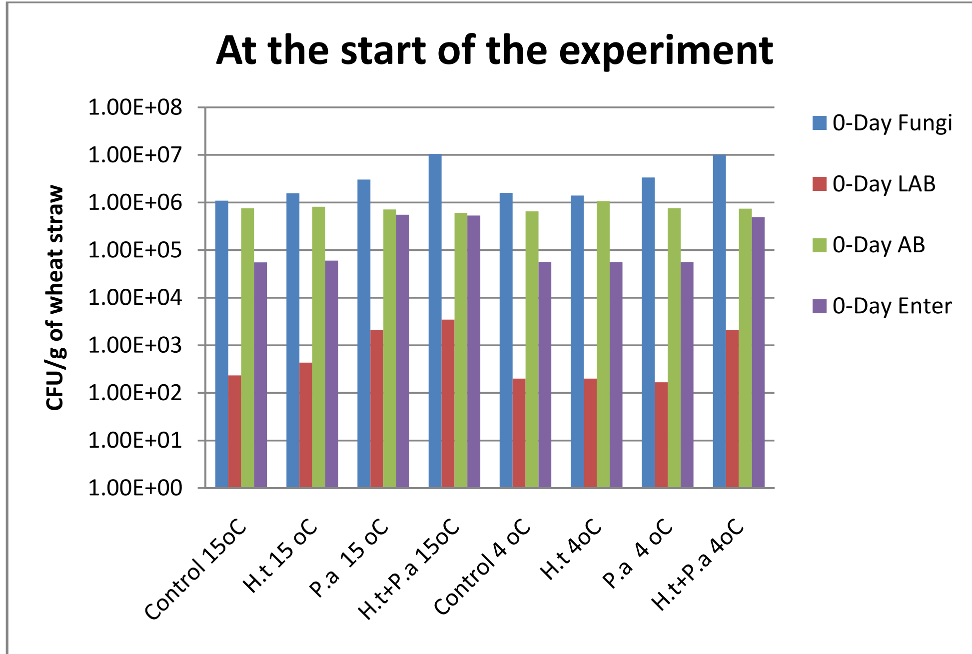


Figure 2: Microbial population at the start of experiment. (Control-Un Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae, and- Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature. LAB-Lactic acid bacteria, AB-Aerobic bacteria, Enter-Enterobacteria)

To get a more detailed understanding of the variation of the microbial load, a chart is plotted (Fig: 3) with the CFU of the control as 1, and all other CFU as the multiple of the control sample. Since the yeast inoculum was in the order of 10^6 and the control sample shows 10^6 fungal CFU/g, one has to assume a 2 fold increase in fungal CFU for all inoculated samples and a 3 fold increment for co culture inoculated samples. The number of fungal CFU was 2 times higher in the *P. anomala* inoculated sample, and approximately 1.2 times in *H. takashimae* inoculated sample, but the co culture fungal CFU was surprisingly higher than the expected value of 3 and showed a 9.6- fold increment. That is the combined effect of *H. takashimae* and *P. anomala* was higher than the individual effect.

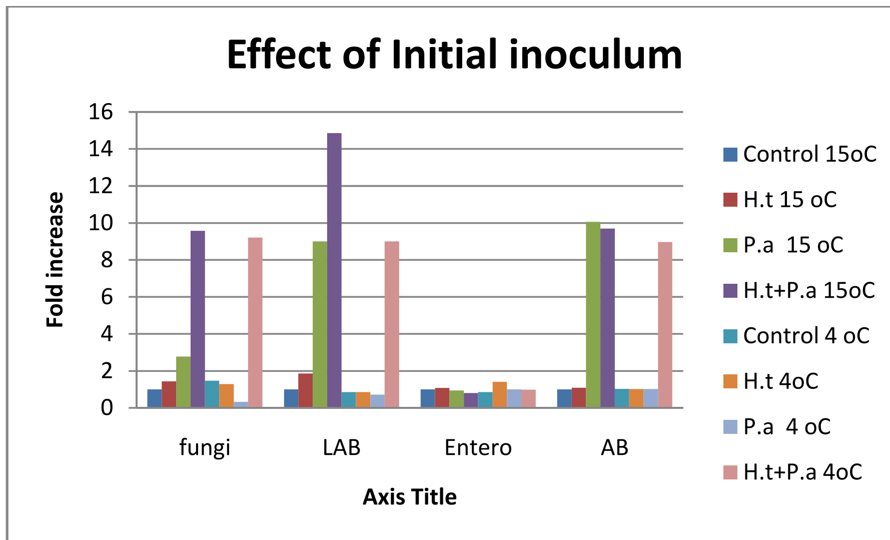


Figure 3: Effect of initial inoculum on microbial load with reference to the control at 15°C. (Control-Un Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae, and- Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature. LAB-Lactic acid bacteria, AB-Aerobic bacteria, Enter-Enterobacteria)

At the same time, one has to assume the same number of Lactic acid Bacterial and Aerobic Bacterial CFU for all the samples, since there is no external addition of any of these organisms and the effect due to the growth of yeast addition may take at least a few hours, which is not possible in t0. LAB CFU were found to be shooted out to a 13 fold increment for the co-culture inoculated samples, and a 9-fold increment for both *H. takashimae* and *P. anomala*. The aerobic Bacteria were 9 times higher than the control sample in both the coculture and Pichia anomala inoculated sample. There is no general answer for this scenario right now, but a general trend of high CFU/g of wheat straw was seen in all co-culture inoculated samples, except for enterobacteria.

4.3.3. *Two week Incubation*

After two weeks of incubation, the control at 15°C in the experiment had approximately 10^6 fungal, and enterobacterial CFU/g, 10^7 aerobic bacteria and zero lactic acid bacteria (Table: 5). Whereas the samples inoculated with *P. anomala* at 15°C showed the highest fungal growth (~10^9 CFU/g) among all the samples (Figure: 3). Also the sample with co-inoculation showed growth up to ~10^8 CFU/g. Low temperature has shown to retard the growth of microorganisms in the all samples (an increment of 10^5 CFU/g to 10^8 CFU/g) except control at low temperature (reduced to 10^4 CFU/g from 10^6 CFU/g at t0).

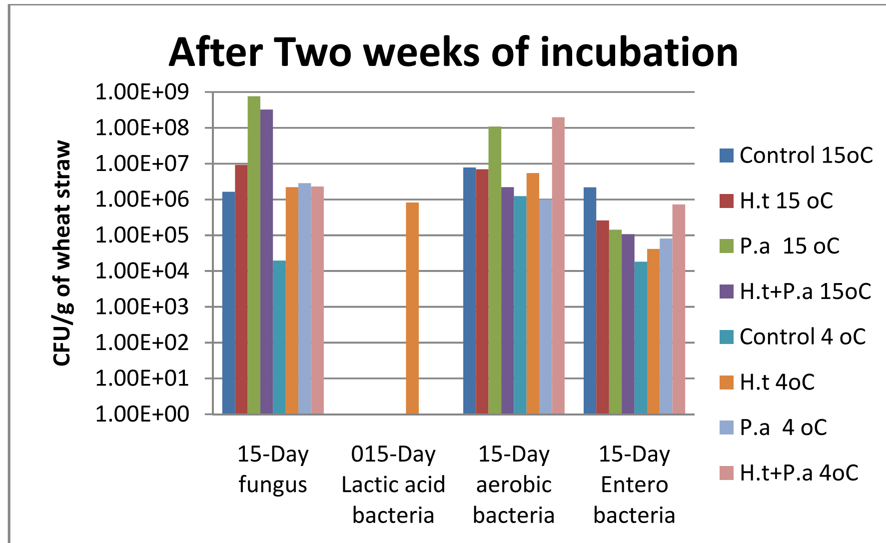


Figure 4 Microbial population after 2 weeks (Control-Un Inoculated wheat straw, H.t- *Holtermanniella takashimae,* P.a- *Pichia anomala*,H.t+P.a-Coculture of *Holtermanniella takashima, and- Pichia anomala.* 15oC represents Higher storage temperature of15°C, 4oC represents low storage temperature of 4°C)

It is interesting to note the enterobacteria count in relation with the yeast population. The biopreservative behavior of *P. anomala* was not observed in the present experiment, instead all the yeast strains found have a slight negative impact on the enterobacteria at 15°C in compared to the control at the same condition.

Aerobic bacteria were in the range of 10^5 CFU/g at the start of incubation. In all the samples aerobic bacterial count increased (Table: 5) suggesting that the aerobic bacteria got benefited by the incubation with yeast.

The lactic acid bacterial population vanished after 2 week of time. There was some growth at one of the plates from *H. takashimae* incubated at low temperature. Since there was no LAB detected in any of the plates or after 1 month, one has to conclude that as a contamination during the handling or during plating.

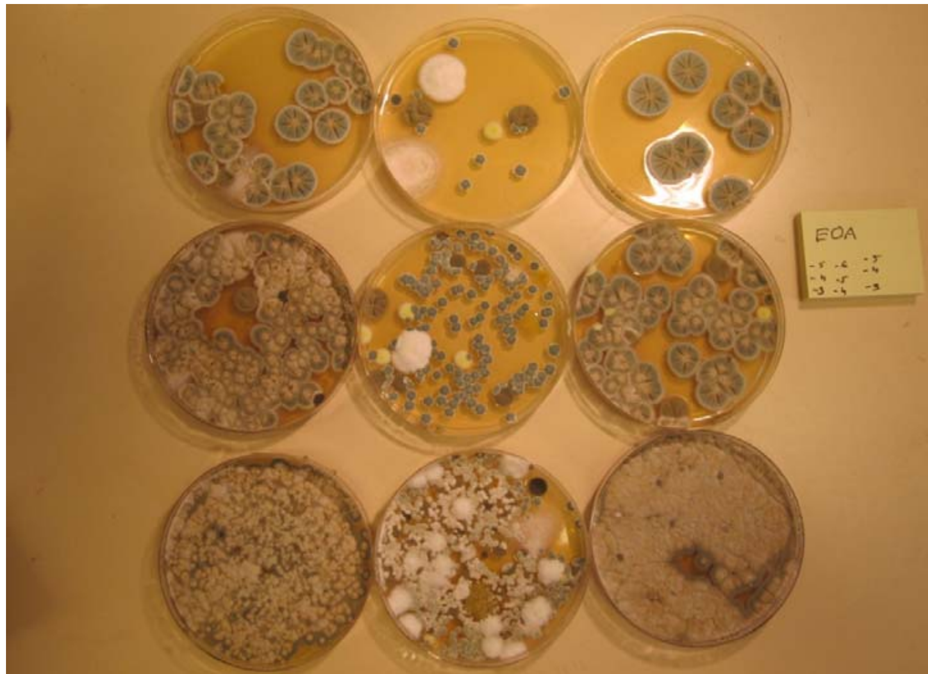


Figure 5: Mold growth of uninoculated control sample at 15oC (YPD plates of different dilutions) after 2 weeks of incubation.

The YPD plate count of uninoculated control (Table: 4) doesn’t represent yeast, instead individual colonies of mold. (Figure: 4). Mold growth was observed in the uninoculated control sample in low temperature also. Whereas all the yeast inoculated samples shows no mold growth. But the co-inoculated sample seems to contained several different populations of yeast. The plates were variably colored. Samples were taken for identification and characterization.

4.3.4. One Month Sample

After 1 month, all the samples showed a decrement in growth (Figure: 5), compared to the 15 days microbial count. Since the trend was common to all the samples, we have to conclude that the nutrients were exhausted in the sample because of the extensive growth. Moreover mold growth was severe in most of the tubes except *P. anomala* inoculated samples. The *P. anomala* seems to inhibit mold in the samples. Co culture of *P. anomala and H. takashimae* also seems to inhibit the mold growth, but it can be also due to the biocontrol activity of *P anomala* alone. The zero in the uninoculated plate count was due to heavy mold growth; it was impossible to count the individual colonies.

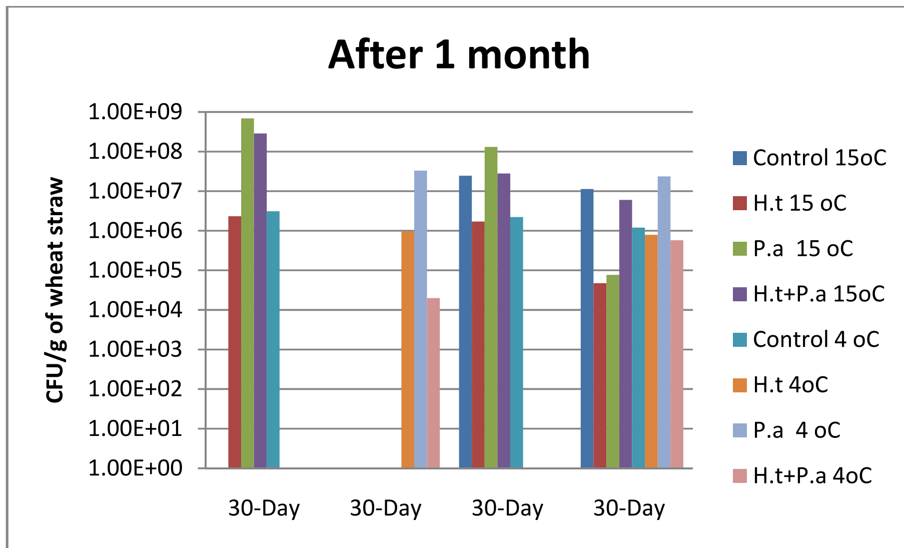


Figure 6: Plate count after 1 month of inoculation (Control-Un Inoculated wheat straw, H.t-*Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae,* and *Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

Even the TGEA plates, which were supposed to support the growth of bacteria, also were heavily populated with mold. The *H. takashimae* inoculated samples also shown to have mold growth, but less severe than the control (Fig: 6). Some yeast was also able to recover from the *H. takashimae* samples after 30 days incubation.

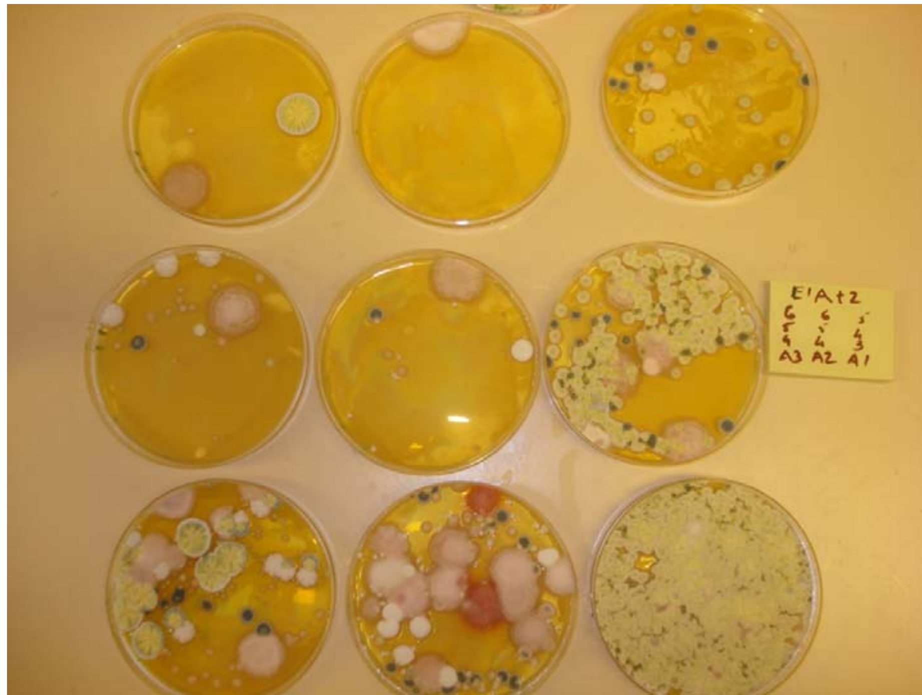


Figure 7: YPD plate count of *H. takashimae* inoculated wheat straw at 15oC (Plates of different dilutions). The different color may represent the occurrence of different fungal species.

There was no lactic acid bacterial growth 15°C or in the control samples, whereas yeast inoculated samples at low temperature some growth of lactic acid bacterial population was observed. In contrast to the *P. anomala* overtaken sample in the

co culture at high temperature, Co culture at low temperature was overtaken by mold

4.4. Yeast growth during storage

Yeast population was the primary target of interest of this project. During the experiment, mold contaminations have inferred with the yeast counting. So the figure (Figure: 6) is not exact for the yeast growth alone. Moreover, the yeasts can belong to different species, other than the inoculated ones. Especially the yeast colonies in the *H. takashimae* inoculated plates were differentially colored and may thus represent different species. This suggests that the *H. takashimae*'s breakdown of cellulose to simple sugars benefits other organisms to proliferate. At the same time, *P. anomala* incubated samples shown high growth with no mold contamination were observed on plates. It may be due to its biocontrol activity [11].

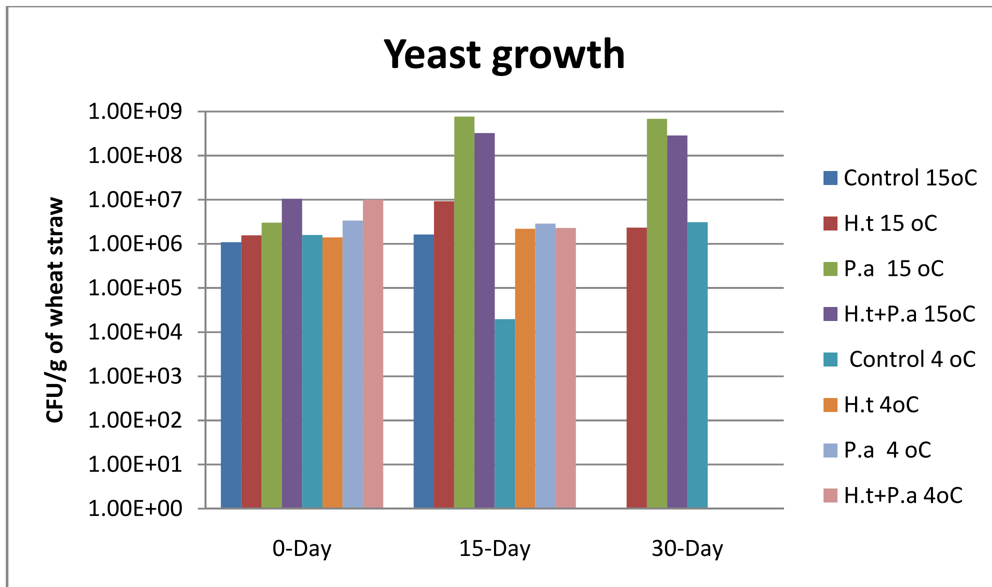


Figure 8: Yeast growth during storage of wheat straw in the mini silos. (Control-Un Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae,* and *Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

The population of yeasts in the uninoculated control was replaced by mold during 2 week incubation time. *P. anomala* showed to have very high growth in all the conditions (10^9 CFU/g Vs 10^6 CFU/g of control). It reached the order of ~10^9 CFU/g in 15°C sample. In all the cases low temperature was shown to retard the growth. After 1 month time the co culture sample at 4°C was found to be dominated by mold, whereas the co culture at high temperature was shown to dominated by a single colony (Colony morphology was same as that of *P. anomala;* samples were preserved for identification).

4.5. Lactic acid Bacteria

As being stated previously, the LAB population dipped to zero in almost all the samples except low temperature incubated *P. anomala* and *H. takashimae* (Fig:8). The reason for such an increment should be investigated further.

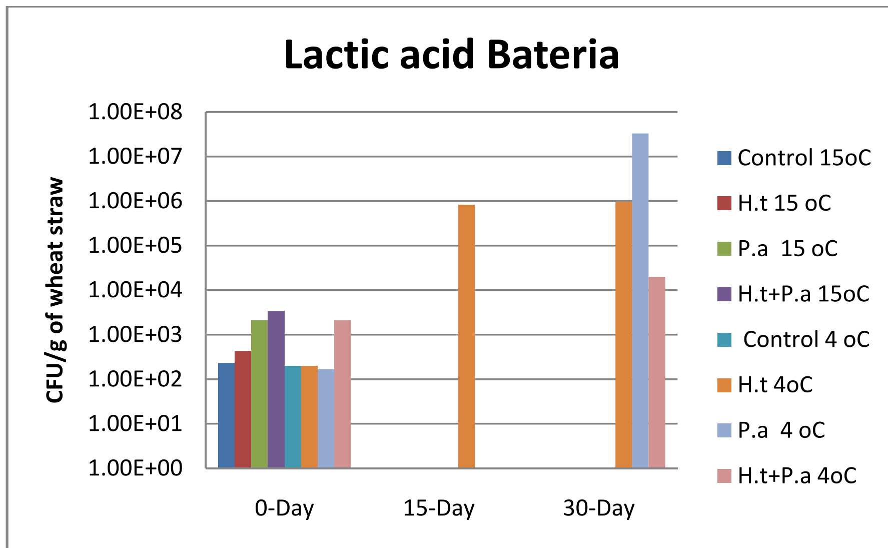


Figure 9: Lactic acid bacterial population during storage in the mini silo. (Control-Un Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae,* and *Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

4.6. Aerobic bacteria

The aerobic bacteria were at the range of 10^{6} CFU/g at the start of incubation. A general trend of an increment of bacteria was observed in all the samples by time (Figure: 9). Highest aerobic bacterial load (10^{8} CFU/g) was observed for co-culture at low temperature. *P. anomala* inoculated wheat straw at 15°C also had high growth compared to the control. After 1 moth of incubation, several plates were unable to count because of mold contamination. Since all the samples had high aerobic bacterial CFU than the control at the same condition, It can be concluded that the yeast growth doesn't have any negative effect on the aerobic bacterial growth.

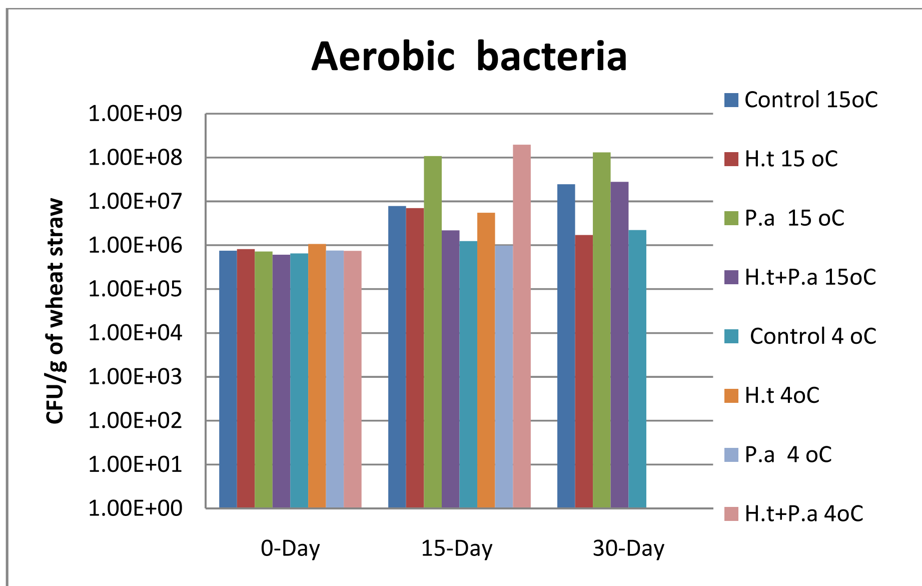


Figure 10: Arerobic bacterial population during the storage in the mini silo. (Control-Un Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala*

inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae,* and *Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

4.7. Enterobacteria

Control at 15°C after 2 weeks of incubation showed higher than 10^6 CFU/g of enterobacteria. Whereas the yeast inoculation and low temperature had a negative effect on the enterobacteria population after 2 weeks of incubation (Fig: 10). After 1 month, *H. takashimae* and *P. anomala* pure culture inoculated sample seems to reduce the enterobacteria. But *P. anomala* and co-culture showed heights enterobacteria population.

The hygienic condition of the sample was in the acceptance level (<10^6 Enterobacteria CFU/g) according to Swedish guidelines[22]in the beginning. But control at 15°C shown to decrease the hygiene and making it well below the standards. The yeast inoculation had some effect, but not a general trend throughout the trials (Figure: 10)

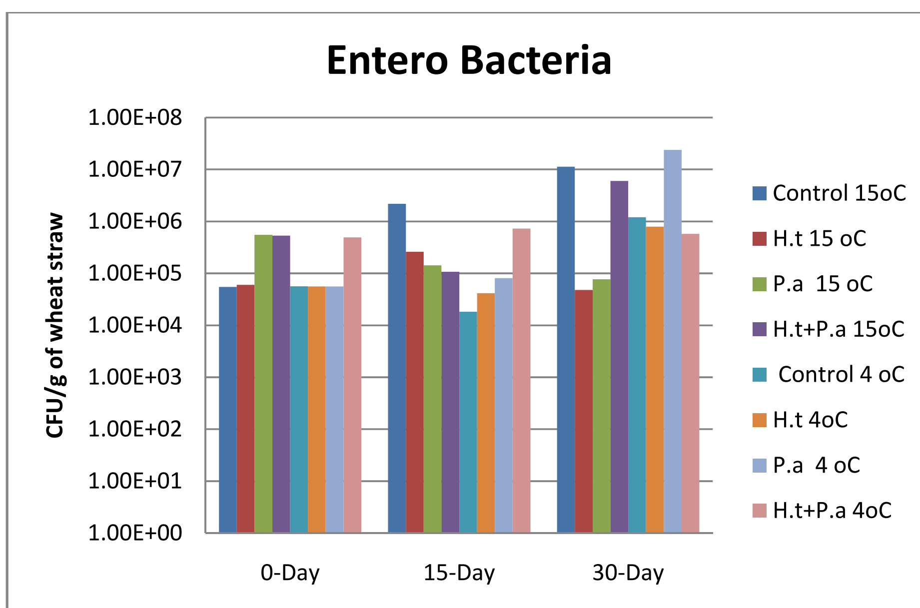


Figure 11: Enterobacterial population during storage. (Control-Un Inoculated wheat straw, H.t-*Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae,* and *Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

4.8. Phenol oxidase activity in stored wheat straw

Phenol oxidases are enzymes which can degrade lignin, and the microbes which are growing on wheat straw, may produce lignin degrading enzymes like Magnesium peroxidase (MnP) lignin peroxidase (LiP) or lacasses. Phenol oxidase activity can be measured by MBTH method. Even though the method is sensitive, the program is unable to calculate the enzyme units/ml for more than 6 significant digits.

A.discolor inoculated samples at 15°C showed the highest (0.002 U/g) enzyme activity among all the samples. Also *H. takashimae* inoculated wheat straw at 15°C showed (0.0014 U/g) and the co-culture of *H. takashimae* and *P. anomala* at low temperature also showed detectable enzyme activity (Table: 3). *A.discolor* being a known lignolytic mold [23], phenol oxidase enzyme activity at 15°C is quite

expected. But at low temperature growth of *A.discolor* may have been retarded and according to the personnel communication with Dr. Leticia Pizzul (Dept of microbiology, SLU) it may be difficult for the mold to grow solely on wheat straw at the beginning stage (Soon after it is transferred from MS-Agar plates). So it is advisable to add little amount of starch material, for the mold to grow on, and to produce lignolytic enzyme. Since the *A.discolor* was a later addition to the project, its growth characteristics were not studied in this work. Most importantly we cannot say how much significant these results are in disturbing the structure of cellulose.

Table 3: Phenol oxidases activity in wheat straw. ***(Control-Non Inoculated wheat straw, H.t- Holtermanniella takashimae inoculated wheat straw, P.a- Pichia anomala inoculated wheat straw, H.t+P.a -Co culture of Holtermanniella takashimae, and Pichia anomala inoculated wheat straw, A.d- A. discolor inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)***

	Units/ g of Wheat straw	
	15°C	4°C
Control	0	0
H. t	0.001447	0
P. a	0	0
H. t +P. a	0	0.00062
A. d	0.00207	0

4.9. Pretreatment

Several pretreatment techniques were tested since the sugar yield of saccharification of non pretreated material was quite low (2,38g of glucose/l of medium) to perform a comparative study (results were not shown). A series of trials simulating steam explosion was done in a bench top autoclave and the glucose produced was compared in HPLC. The maximum amount of glucose produced was 6.21 g/L and the process parameters were difficult to control (sudden release of pressure and initial heating time varies from sample to sample). The dilute acid treatment was easy to perform at laboratory scale and was able to produce up to 14 g/L Glucose. A simple biological toxicity test were performed later to find out the extent of severity of our the dilute acid treatment (see section 4.12.4)

4.10. Saccharification

Saccharification was done in the same bottles where we have done the dilute acid treatment. The samples after acid treatment were sterile, and since the saccharification is highly vulnerable for contamination, it is important to keep the sterility. The pretreated samples were at very low pH (~1) so the pH was increased by the addition of 25 % NaOH, and Citrate –buffer was added to give a buffer for the Accellerase™ 1000 enzyme. Unfortunately some of the 15°C samples fell down from the shaker and are not included in the final results. But we were able to get more than one parallel for each sample.

4.11. Glucose Production

Control at 15°C showed highest glucose yield (13.1 g/L) compared to the fungi inoculated samples. And the difference between control and inoculated sample was quite low (~2 g/L) (Figure: 11). It indicates that the tested organisms had only little effect on pretreatment efficiency or apparently growth of microorganisms reduced the yield of sugar. Since the hypothesis was high ethanol production for the samples with highest microbial growth, the results was not promising.

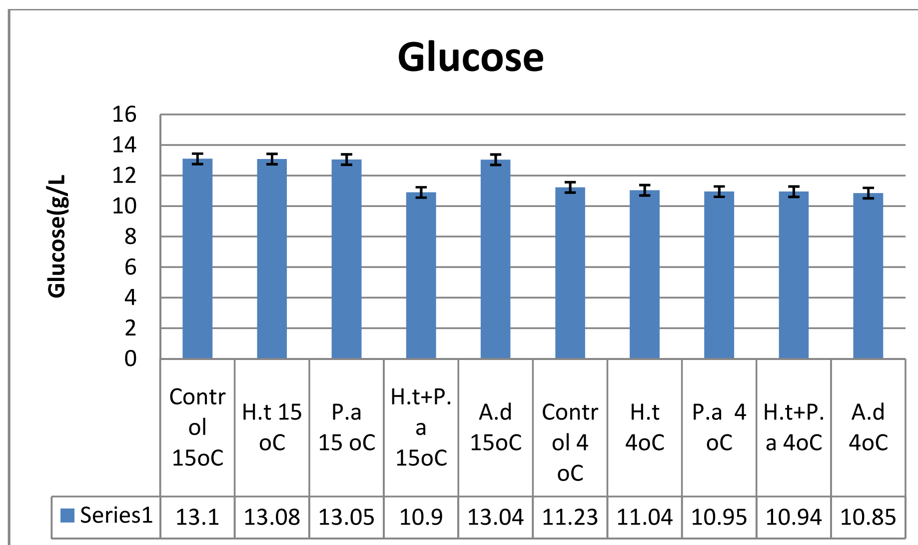
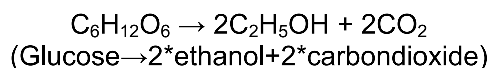


Figure 12: Amount of glucose in the hydrolysate before start of fermentation. (Control-Non Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae,* and *Pichia anomala* inoculated wheat straw, A.d- *A. discolor* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

4.12. Fermentation studies

The yeast cells produce ethanol under anaerobic situation, when they convert the pruvate (which is the break down product of glucose) into ethanol for gain of 2 molecules NAD+, which is needed for the continuation of the energy yielding glycolsis process. The overall reaction of ethanol fermentation can be represented by



$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$
(Glucose→2*ethanol+2*carbondioxide)

The fermentation medium was initially analyzed for glucose, acetate and ethanol. The ethanol productions varied in different samples. The highest yield was for *P. anomala* at 15°C (6.87 g/L) Vs 6.47 g/L of uninoculated control

4.12.1. Ethanol Production

The HPLC analysis of fermented medium shows an inverse co-relation between amounts of glucose detected previously. The inoculated samples gave better ethanol production than the uninoculated-control sample (figure 12). Highest value for ethanol was *A. discolor* at 15°C (6.85 g/L), where as the co-inoculated sample (5.83 g/L) gave the least ethanol production value than the control. At low temperature (4°C) *P. anomala* inoculated wheat straw showed the highest ethanol production (6.23 g/L). But the overall ethanol yield was lower in 4°C

stored samples than the 15°C stored samples. It may be an indication of low ethanol production due to low structural damage/ low growth of microorganisms (figure 3&4).

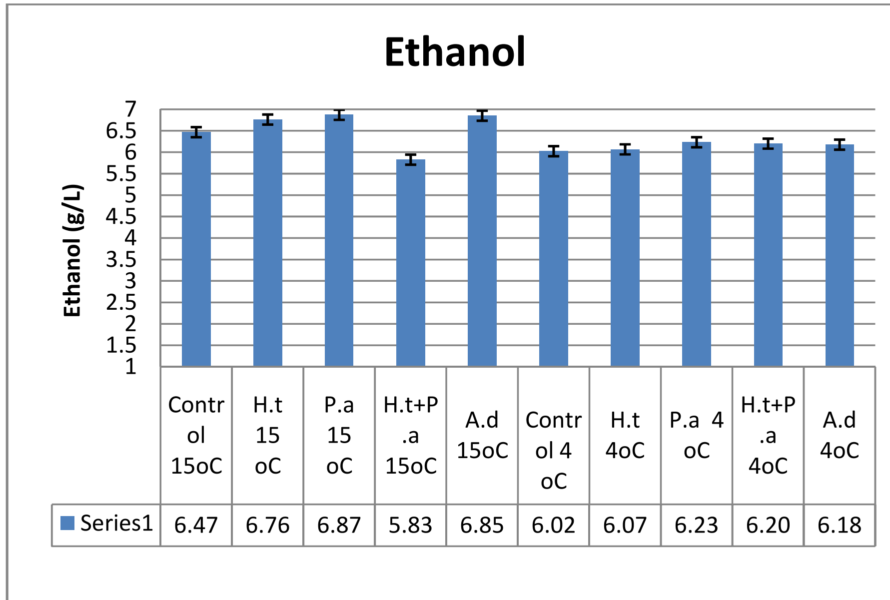


Figure 13: Amount of ethanol after 3 days of fermentation. (Control-Non Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw*,* P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae,* and *Pichia anomala* inoculated wheat straw, A.d- *A. discolor* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

4.12.2. Yield Factor

It is theoretically impossible to produce 6.18g of ethanol from a hydrolysate containing 10.8g of glucose, unless there are other sources of fermenting sugars left in the medium. Since the fermenting medium was already filtered out to remove non hydrolyzed solid factors, the only possible scenario is the presence of soluble polysaccharides left over from the saccharification step, which were further converted to sugars during fermentation step. The yields of ethanol Vs glucose have been shown in the graph below, showing highest yield at 57 % for *P. anomala* at 4°C (figure: 13). It also raises a question why the yield factor is less for uninoculated sample.

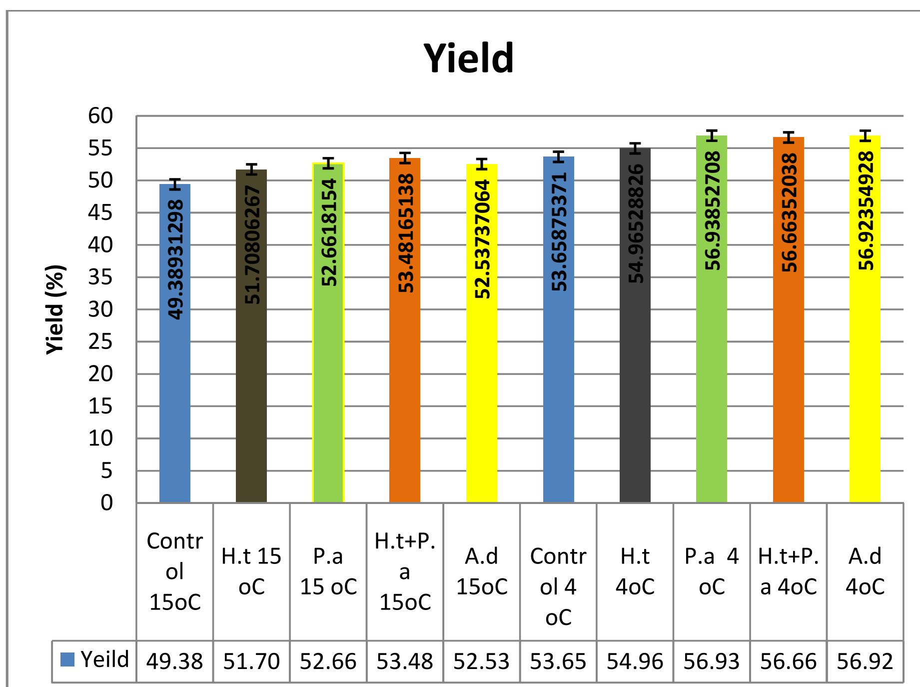
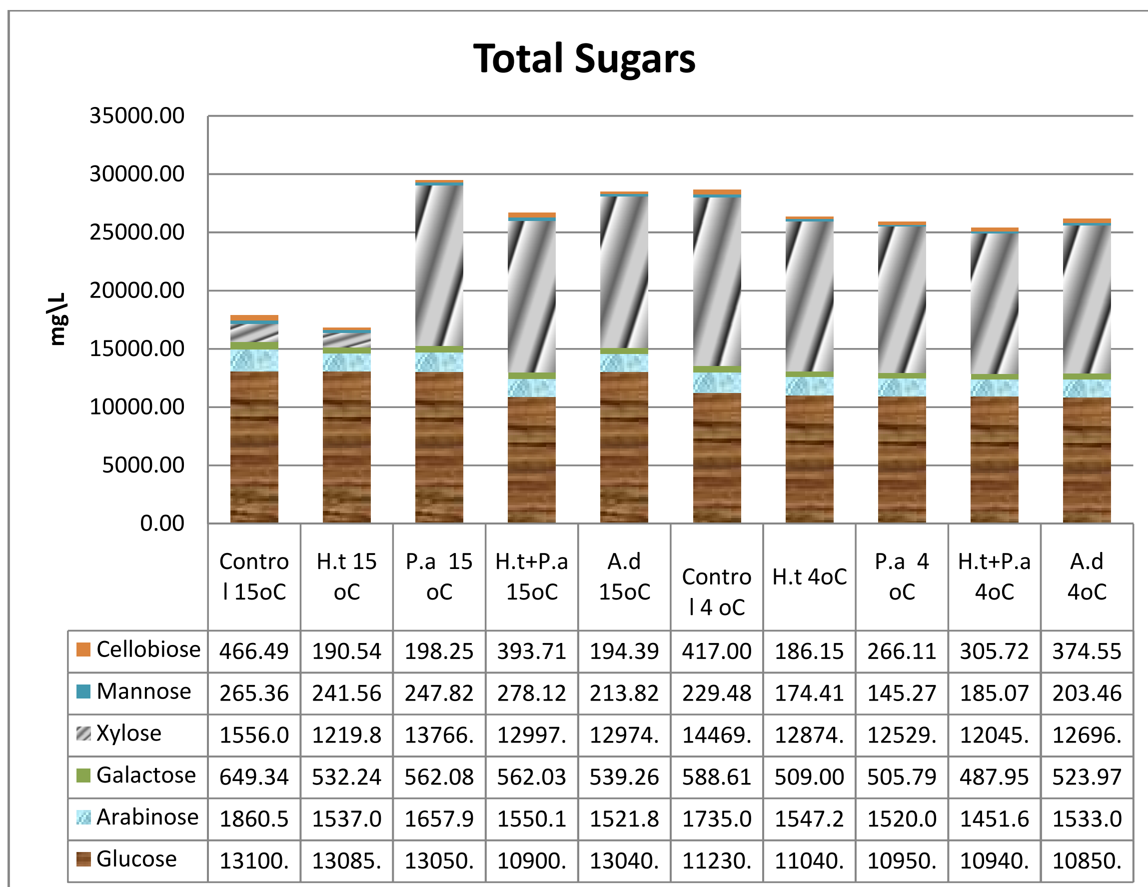


Figure 14: Yield of ethanol from amount of glucose before the start of fermentation. (Control-Un Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw*,* P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae, and- Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

4.12.3. Other sugars

Since the yield factor is very high (Figure: 13), we have to assume that, the hydrolysate contains other possible simple sugars which can be fermented by the yeast, or other soluble poly saccharides which can be converted to glucose by the still active enzyme. So an investigation was done to measure other simple sugars like xylose, arabinose, mannose and cellobiose in a Dionex Anion-exchange high-performance liquid chromatography (HPAEX) system.



	Control 15oC	H.t 15 oC	P.a 15 oC	H.t+P.a 15oC	A.d 15oC	Control 4 oC	H.t 4oC	P.a 4 oC	H.t+P.a 4oC	A.d 4oC
Cellobiose	466.49	190.54	198.25	393.71	194.39	417.00	186.15	266.11	305.72	374.55
Mannose	265.36	241.56	247.82	278.12	213.82	229.48	174.41	145.27	185.07	203.46
Xylose	1556.0	1219.8	13766.	12997.	12974.	14469.	12874.	12529.	12045.	12696.
Galactose	649.34	532.24	562.08	562.03	539.26	588.61	509.00	505.79	487.95	523.97
Arabinose	1860.5	1537.0	1657.9	1550.1	1521.8	1735.0	1547.2	1520.0	1451.6	1533.0
Glucose	13100.	13085.	13050.	10900.	13040.	11230.	11040.	10950.	10940.	10850.

Figure 15: Simple sugars produced in the hydrolysate after 3 days of saccharification. (Control-Un Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw*,* P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae, and- Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

There was a significant difference between the amounts of xylose produced among various samples. An 88% higher xylose yield was obtained for *P. anomala* inoculated sample than that of control. It is interesting to note that the amount of glucose was lowest for the co culture (10.9 g/L) but the total sugars, and ethanol yield was not affected by the low glucose concentration.

4.12.4. Toxicity of hydrolysate

It is interesting to find out the extent of toxicity of the hydrolysate generated by the primitive pretreatment step. But quantification of such compounds is quite difficult and costly. So a biological feasibility test was conducted. Since it was shown that *S. cerevisiae* can grow in 100% hydrolysate; *P. stipitis*, a yeast species known for its vulnerability to inhibitors, which also can ferment on xylose and an alternative fermentation yeast *D. bruxellensis* (at OD 2 and 1) was inoculated into a test fermentation. Since the incubation time was quite long for *D.bruxellensis*, ethanol concentration was measured after 1 week.

Both the species were able to grow in 100 % medium (un inoculated wheat straw hydrolysate). *D. bruxellensis* produced little more ethanol (6.7 g/L) than the previous *S. cerevisiae* fermentation. *P. stipitis* produced only 4.8 g/L ethanol (may be due to the long incubation time of 1 week and air diffusion through the needle hole in the fermentation cap). Since both the species was able to grow in concentrated medium, and able to produce ethanol, it is obvious that the used

pretreatment produces much less fermentation inhibitors than the previous hydrolysate obtained by steam explosion [21] and quite vulnerable yeast strains were able to grow with fairly good ethanol yield.

A back calculation of the dilute acid pretreatment showed that, the current H_2SO_4 concentration was only 0.66 % instead of the targeted 0.75 % because of the moisture content in the sample. So we can conclude that, our pretreatment was less severe and produced almost equal amount of sugar to that of commercial harsh pretreatment step [21] without production of inhibitors.

4.12.5. Statistical significance

Since one of the 3 bottles of the control at 15°C got fall down from the shaker, it created an unbalanced distribution, and the program was unable to calculate the statistical significance with the data. So the control at 4°C was used as a reference for calculating the significance of ethanol production. Moreover, it is logical to compare the ethanol production of high microbial growth with samples having less growth.

The higher yield obtained by the co-culture (2.8 %) and the *A.discolor* (2.5 %) at low temperature was statistically significant compared to the wet non inoculated wheat straw at low temperature.

5. CONCLUSION AND FUTURE PROSPECTIVE

Even though the difference in ethanol production was marginal(2.8 % higher ethanol produced by the Co-culture at 4°C than the control at the same temperature), the theoretically impossible yield (57 % conversion of glucose to ethanol) suggests that, there were more unconverted soluble sugar in the *A.discolor* inoculated sample than the non inoculated sample. That is, the saccharification was not efficient enough to convert all the cellulose to glucose, instead, it reached a certain value and got inhibited for some reasons. When the *S. cerevisiae* started ethanol fermentation, the soluble sugars got converted to glucose by the still active enzyme in the medium.

The comparison of total sugar yield indicates that wheat straw inoculated with *P. anomala* at 15°C produce approximately double the quantity of simple sugars that of control sample. Moreover, there was more xylose than glucose, which the common baker's yeast cannot utilize. So it is suggested to use other fermenting yeast like *P. stipitis* which can utilize xylose also. In order to meet the objective of the current project, it is highly advisable to run the projects with more enzymes until all the cellulose is converted to sugar monomers or a simultaneous saccharification and fermentation to fully utilize the cellulose to ethanol. Greater yield difference is between uninoculated sample at 4°C and that of lignolytic mold at higher temperature (12.3 %), suggests that the storage is more efficient where microorganisms capable of degrading cellulose are more active. Moreover in future it is more meaningful to compare the results with dry non stored wheat straw with inoculated stored samples.

6. ACKNOWLEDGEMENT

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Appendix

Table 4: Microbial enumeration of initial sample-after water activity normalization and inoculation with the respective yeast sample. (Control-Non Inoculated wheat straw, H.t-*Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae, and- Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

	Sample	Name	YPD-0	MRS-0	TGEA-0	VRBG-0
Incubated at 15°C	Control	E0A1t0	1.10E+06	5.00E+02	6.80E+05	5.80E+04
	Control	E0A2t0	9.80E+05	1.00E+02	8.10E+05	5.00E+04
	Control	E0A3t0	1.20E+06	1.00E+02	7.80E+05	5.70E+04
	H.t	E1A1t0	1.03E+05	0.00E+00	1.01E+06	6.00E+04
	H.t	E1A2t0	3.90E+06	7.00E+02	6.40E+05	6.60E+04
	H.t	E1A3t0	6.90E+05	6.00E+02	7.90E+05	5.40E+04
	P.a	E2A1t0	2.40E+06	5.90E+03	7.80E+05	4.80E+05
	P.a	E2A2t0	4.50E+06	1.00E+02	6.00E+05	7.20E+05
	P.a	E2A3t0	2.20E+06	3.00E+02	7.70E+05	4.60E+05
	H.t +P.a	E3A1t0	1.16E+07	9.70E+03	6.60E+05	4.60E+05
	H.t +P.a	E3A2t0	8.40E+06	0.00E+00	6.30E+05	6.30E+05
	H.t +P.a	E3A3t0	1.14E+07	7.00E+02	5.40E+05	5.10E+05
Incubated at 4°C	Control	E0B1t0	2.10E+06	1.00E+02	6.50E+05	6.50E+04
	Control	E0B2t0	1.80E+06	4.00E+02	6.50E+05	4.40E+04
	Control	E0B3t0	9.00E+05	1.00E+02	7.60E+05	6.00E+04
	H.t	E1B1t0	2.40E+06	0.00E+00	1.12E+06	6.00E+04
	H.t	E1B2t0	6.10E+05	1.00E+02	1.00E+06	5.00E+04
	H.t	E1B3t0	1.21E+06	5.00E+02	1.08E+06	5.80E+04
	P.a	E2B1t0	2.02E+06	4.00E+02	7.60E+05	4.40E+04
	P.a	E2B2t0	4.10E+06	0.00E+00	7.60E+05	6.60E+04
	P.a	E2B3t0	4.00E+06	1.00E+02	7.60E+05	5.80E+04
	H.t +P .a	E3B1t0	1.37E+07	5.80E+03	6.90E+05	4.70E+05
	H .t +P .a	E3B2t0	2.51E+06	0.00E+00	7.70E+05	5.10E+05
	H.t +P.a	E3B3t0	1.40E+07	5.00E+02	7.70E+05	5.00E+05

Table 5: Microbial population at 2 week. (Control-Non Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae, and- Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

	Sample	Name	YPD-15	MRS-15	TGEA-15	VRBG-15
Incubated at 15°C	Control	E0A1t1	8.00E+05	0.00E+00	6.30E+06	2.00E+06
	Control	E0A2t1	1.40E+06	0.00E+00	9.40E+06	2.30E+06
	Control	E0A3t1	2.70E+06	0.00E+00	mold	2.24E+06
	H.t	E1A1t1	9.10E+06	0.00E+00	7.20E+06	1.28E+05
	H.t	E1A2t1	9.90E+06	0.00E+00	7.80E+06	1.10E+05
	H.t	E1A3t1	8.80E+06	0.00E+00	6.00E+06	5.40E+05
	P.a	E2A1t1	7.70E+08	0.00E+00	1.66E+08	1.41E+05
	P.a	E2A2t1	8.00E+08	0.00E+00	1.69E+03	1.68E+05
	P.a	E2A3t1	7.20E+08	0.00E+00	1.60E+08	1.18E+05
	H.t +P .a	E3A1t1	1.96E+08	0.00E+00	2.20E+06	1.07E+05
	H.t +P.a	E3A2t1	3.60E+08	0.00E+00	1.80E+06	8.00E+04
	H.t +P.a	E3A3t1	4.20E+08	0.00E+00	2.60E+06	1.34E+05
Incubated at 4°C	Control	E0B1t1	2.30E+04	0.00E+00	1.18E+06	1.70E+04
	Control	E0B2t1	1.40E+04	0.00E+00	1.20E+06	1.10E+04
	Control	E0B3t1	2.20E+04	0.00E+00	1.33E+06	2.70E+04
	H.t	E1B1t1	3.90E+05	9.40E+05	8.40E+06	4.00E+04
	H.t	E1B2t1	2.60E+06	1.53E+06	1.50E+06	1.16E+04
	H.t	E1B3t1	3.60E+06	0.00E+00	6.60E+06	7.30E+04
	P.a	E2B1t1	5.10E+06	0.00E+00	9.80E+05	8.00E+04
	P.a	E2B2t1	1.80E+06	0.00E+00	7.50E+05	6.90E+04
	P.a	E2B3t1	1.70E+06	0.00E+00	1.23E+06	9.40E+04
	H.t +P .a	E3B1t1	1.60E+06	0.00E+00	2.24E+08	1.08E+06
	H.t +P .a	E3B2t1	2.90E+06	0.00E+00	2.00E+08	4.40E+05
	H.t +P .a	E3B3t1	2.40E+06	0.00E+00	1.64E+08	6.60E+05

Table 6: Microbial population after 1 month. 'M' represents a non zero value, due to high mold contamination in the counting plates. (Control-Non Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co

culture of *Holtermanniella takashimae, and- Pichia anomala* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

	Sample	Name	YPD-30	MRS-30	TGEA-30	VRBG-30
Incubated at 15°C	Control	E0A1t2	M	0.00E+00	3.40E+07	1.12E+07
	Control	E0A2t2	M	0.00E+00	2.22E+07	9.80E+06
	Control	E0A3t2	M	0.00E+00	1.77E+07	1.30E+07
	H.t	E1A1t2	3.70E+06	0.00E+00	3.60E+06	8.30E+04
	H.t	E1A2t2	1.30E+06	0.00E+00	5.40E+05	6.00E+04
	H.t	E1A3t2	2.00E+06	0.00E+00	1.00E+06	1.40E+01
	P.a	E2A1t2	9.10E+08	0.00E+00	1.32E+08	8.80E+04
	P.a	E2A2t2	4.88E+08	0.00E+00	1.40E+08	6.50E+04
	P.a	E2A3t2	6.63E+08	0.00E+00	1.22E+08	7.80E+04
	H.t +P .a	E3A1t2	2.48E+08	0.00E+00	3.44E+07	4.80E+06
	H.t +P.a	E3A2t2	3.84E+08	0.00E+00	2.94E+07	3.30E+06
	H.t +P .a	E3A3t2	2.28E+08	0.00E+00	2.04E+07	9.90E+06
Incubated at 4°C	Control	E0B1t2	2.80E+06	0.00E+00	8.00E+05	1.84E+06
	Control	E0B2t2	3.00E+06	0.00E+00	2.40E+06	6.60E+05
	Control	E0B3t2	3.50E+06	0.00E+00	3.50E+06	1.10E+06
	H.t	E1B1t2	8.00E+05	0.00E+00	9.10E+05	1.20E+04
	H.t	E1B2t2	1.11E+06	0.00E+00	8.80E+05	8.00E+03
	H.t	E1B3t2	9.80E+05	0.00E+00	5.80E+05	3.00E+04
	P.a	E2B1t2	4.20E+07	0.00E+00	3.20E+07	2.10E+04
	P.a	E2B2t2	3.80E+07	0.00E+00	2.72E+07	2.80E+04
	P.a	E2B3t2	1.90E+07	0.00E+00	1.20E+07	2.00E+04
	H.t +P .a	E3B1t2	M	0.00E+00	6.30E+05	2.80E+04
	H.t +P .a	E3B2t2	M	0.00E+00	5.40E+05	1.30E+04
	H.t +P.a	E3B3t2	2.00E+04	0.00E+00	5.50E+05	1.00E+04

Table 7: HPLC analysis of wheat straw hydrolysate after 3 days enzyme treatment with Acerylase 1000. (Control-Non Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae,* and *Pichia anomala* inoculated wheat straw, A.d- *A. discolor* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

		Before fermentation			
	Sl No	**Sample**	**Glucose (g/L)**	**Acetate (g/L)**	**Ethanol (g/L)**
Incubated at 15°C	1	Control	13.1±0	1.25±0	0
	2	*H.t*	13.085±0.120	1.28±0.05	0
	3	*P.a*	13.05±0.34	1.28±0.02	0
	4	*H.t +P.a*	10.9±2.20	1.25±0.02	0
	5	*A. d*	13.045±1.56	1.25±0.09	0
Incubated at 4°C	6	Control	11.23±0.75	1.66±0.03	0
	7	*H.t*	11.043±0.04	1.56±0.01	0
	8	*P.a*	10.95±0.10	1.56±0.05	0
	9	*H.t +P.a*	10.94±0.056	1.52±0.08	0
	10	*A. d*	10.856±0.14	1.60±0.02	0

Table 8: HPLC analysis Fermentation medium after 3 days of fermentation with *S. cerevisiae* at *30°C. (Control-Non Inoculated wheat straw, H.t- Holtermanniella takashimae inoculated wheat straw, P.a- Pichia anomala inoculated wheat straw, H.t+P.a -Co culture of Holtermanniella takashimae, and Pichia anomala inoculated wheat straw, A.d- A. discolor inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)*

After fermentation						
	Sl No	**Sample**	**Glucose (g/L)**	**Acetate (g/L)**	**Ethanol (g/L)**	**Yield (%)**
Incubated at 15°C	1	Control	0	1.19±0	6.47±0	49.38931
	2	*H.t*	0	1.21±0.28	6.76±0.07	51.70806
	3	*P.a*	0	1.2±0.21	6.87±0.06	52.66182
	4	*H.t +P.a*	0	5.8295±0.042	5.82±1.28	53.48165
	5	*A. d*	0	1.21±0.04	6.85±0.59	52.53737
	6	Control	0	1.6±0.05	6.02±0.01	53.65875

	7	*H.t*	0	1.53±0.052	6.07±0.09	54.96529
	8	*P.a*	0	1.53±.00	6.23±0.23	56.93853
	9	*H.t +P.a*	0	1.46±0.65	6.20±0.10	56.66352
	10	*A. discolor*	0	1.65±0.31	6.18±0.07	56.92355

Table 9: HPLC analysis of wheat straw hydrolysate in Dionex system. (Control-Non Inoculated wheat straw, H.t- *Holtermanniella takashimae* inoculated wheat straw, P.a- *Pichia anomala* inoculated wheat straw, H.t+P.a -Co culture of *Holtermanniella takashimae,* and *Pichia anomala* inoculated wheat straw, A.d- *A. discolor* inoculated wheat straw. 15°C represents Higher storage temperature, 4°C represents low storage temperature.)

	Sample	**Arabinose(mg/L)**	**Galactose(mg/L)**	**Xylose(mg/L)**	**Manose(mg/L)**	**Cellobiose(mg/L)**
Incubated at 15°C	Control	1860.55	649.3448	15576.08	265.369	466.493
	H.t	1537.04±7.13	532.24±2.89	1219.86±308.41	241.56±36.65	190.54±11.67
	P.a	1657±113.07	562.08±42.44	13766.81±882.51	247.82±54.10	198.25±1.19
	H.t +P.a	1550.16±12.6	562.03±11.28	12997.60±208.69	278.12±4.6	393.71±44.63
	A. d	1521.84±94.72	539.29±19.26	12974.96±893.056	213.82±19.34	194.39±16.49
Incubated at 4°C	Control	1735.04±180.63	588.61±65.65	14496.54±1562.527	229.48±31.99	417±20.56
	H.t	1547.27±79.29	509±41.78	12874.78±485.26	174.417±42.28	186.15±3.10
	P.a	1520.04±77.52	505.79±34.08	12529.04±686.41	145.27±126.02	266.11±72.05
	H.t +P.a	1451.62±71.75	487.95±16	12045.91±615.02	185.07±13.54	305.72±7.12
	A. d	1533.07±29.04	523.97±18.33	12696.61±215.97	203.465±17.52	374.55±6.72