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ABSTRACT

Production of ethanol using cellulosic material as feedstock is crucial for sustainable fuel ethanol production.However a production process based on cellulosic biomass involves several energy and cost intensive steps like storage of biomass, pretreatment, hydrolysis and fermentation, where pretreatment is the energy intensive and troublesome step. This project aimed for an integration of storage and pretreatment step, to get more energy efficiency and more ethanol yield. In the present investigation wheat strawwas used as a model and was stored in moist conditions with different fungal species (*Pichia anomala, Pichia stipitis*, and *Anthracophyllum discolor)* inoculated separately in mini-silos for 1 month at 15°C and 4°C. Simultaneous saccharification and fermentation was carried out afterthe storage period and ethanol yields were compared with dry wheat straw as a control.A7.52 % higher ethanol yield (compared to the dry wheat straw) was obtained from wheat straw incubated by *P. anomala* at 15°C, and 6.87 % higher ethanol yield from *P.stipitis* inoculated wheat straw incubated at 4°C showed ISP can result in increasing the ethanol yield. Also it was obvious from the study that, the release of sugar from integrated storage and pretreatment (ISP) sample was faster than from the traditional sample. The higher concentration of non-fermentable sugars (eg: xylose, arabinose, mannose etc.) left during fermentation of ISP samples indicate that the ISP process causes more structural damage to the cellulosic substances and produces more sugar release than the control. Moreover *P. anomala* and *P.stipitis* showed a biocontrol activity during moist storageby preventing growth of other fungi and enterobacteria in the wheat straw during the one month incubation. In conclusion, ISP acted as an efficient method of storage and resulted in higher ethanol yield.

Key words*: Biofuel; wet storage; Biocontrol; Dilute acid pretreatment. Simultaneous saccharification and fermentation; Pichia anomala; Pichia stipitis; Anthracophyllum discolor.*

POPULAR ABSTRACT

Fuel ethanol is one of the best choices of liquid fuel replacement for petroleum. However the productions of ethanol from starch and sugar materials are not sustainable since it may deplete our food supply. One way to overcome this issue is developing a technology to produce ethanol from abundant cellulosic material like wood chips and agricultural residues. But production of ethanol from hard and crystalline cellulose requires an energy intensive thermo chemical pretreatment step, which disturbs the crystal nature of cellulose and softens the biomass, making the access ofhydrolyzing enzymes moreeasy. This project deals with an ingenious way of integrating the pretreatment step with biomass storage (Integrated storage and pretreatment-ISP). Inoculating the wheat straw with yeasts species *P. anomala*, *P. stipitis* and a lignolytic mold *A. discolor* softened the material, which subsequently reduced the need of severe thermo chemical treatments. Moreoverunder certain ISP conditions a substantially increased ethanol yield (7.52 %) compared to the dry control was obtained. Also the ISP treated sample showed faster release of sugar, producingmore ethanol in less time. *P. anomala* and *P. stipitis* showed inhibition of enterobacteria and molds in moist storage situation, making ISP with these organisms an efficient storage method. The results from this study indicate that ISP has a greater potential to decrease the pretreatment intensity and increases the ethanol yield in lignocellulosic based ethanol production.

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1. INTRODUCTION

Ethanol is a widely accepted and currently used fuel alternative or fuel blendof microbial origin. But currently 90% of all fuel ethanol is derived from sugar or starch based raw materials[1], which can cause competition with food. Therefore it is important to develop an ethanol production technique from non food materials like agricultural residues and forest remains, mainly known as lignocellulosic material. But the lignocellulosic materials are not readily fermentable, since the fermentation yeast cannot convert the complex polysaccharides into ethanol[1]. For this reason a suitable conversion step has to be applied to break the complexstructure of the polysaccharide into much simpler structures (softening) for the easy access of the saccharification enzymes where the enzymes convert the polysaccharides into fermentable sugars. The first step of this conversion process is commonly known as pretreatment of biomass, where severe thermo chemical treatment is used to break the complex chemical structure of lignocellulose[2]. During this harsh and severe thermo chemical treatment, phenolic compounds(Lignin) and sugars in the plant material get converted to toxic chemical substances, which further inhibit the fermentation process[3-4]. Therefore it is important to reduce the severity of pretreatment, but at the same time produce fermentable sugars.

Hatakka 1983[5] proposed a biological pretreatment of wheat straw using white rot fungi instead of thermo chemical pretreatment. Even though he was able to produce high ethanol yield, the requirement for sterile incubation of biomass for 5 weeks proves the technique non economic and practically impossible. Hatakka also tested a semi bio-chemical treatment, with a reduced time of biological treatment, and subsequent thermo chemical pretreatment. But some sugars released by the initial biological treatment were converted to inhibitors in the later chemical treatment.Also a masking effect (The yield difference produced by the biological treatment was masked by the subsequent chemical treatment) was observed, making the process less feasible. On the other hand Hatakka has proved that, microorganisms can be a suitable environmentally friendly alternative for thermo chemical pretreatment, if suitable time and conditions are provided.Cellulose degradation is a natural process in nature, with the symbiotic cooperation of many microorganisms[6]. Haruta et al. 2002 constructed a stable microbial community with high cellulose degradation ability and demonstrated a 60% degradation of rice straw within 4 days at 50°C[7]. But for a lignocellulosic pretreatment for ethanol production, such strong degradation is not a desired characteristic, since it will reduce the ethanol yield. In conclusion, an ideal biological pretreatment should beuseful in reducing the lignin content and the crystallinity of cellulose with increase in the surface area for the easy access of enzymes without prior need of any sterilization.

1.1. Energy efficient Storage

During commercial production of ethanol, there will be a gap between the feedstock production phase and ethanol production. The supply of biomass for the ethanol production industry should be endless, whereas the harvest of biomass occurs only during the suitable season. That is the biomasses have to be stored for weeks to months. The most common practice of material conservationis drying. But in countries like Sweden, where there is less

sun during the harvest season the drying is done by hot air dryers, which need high energy input for the process. The principle of dry storage is preventing unwanted microbial growth on the material by reducing the moisture content [8]. Druvefors et al. 2002 showed that Biopreservation with *Pichia anomala* is efficient in reducing spoilage mold in moist wheat grain storage[9]. Moreover Passoth et al. 2009 has found that airtight storage of moist wheat grain improves the bioethanol yield allowing the enzymes easy access to the starch. At the same time, addition of biocontrol yeast *P. anomala* or addition of other cellulase enzymes did not further improve the ethanol yield[10]. In otherwords storing the biomass in moist condition will reduce the process energy needed for storage of material, and inoculation of biocontrol yeast may help to reduce the material loss by unwanted microbial growth (or degradation).

1.2. Integrated Storage and Pretreatment

The concept of integrated storage and pretreatment is based on breaking down the crystal structure of biomass during the time of biomass storage. Like thermo chemical pretreatment,randomly growing microorganisms generate partial breakage of crystal nature of celluloseimprovingthe accessibility of enzyme[11]. In a traditional ethanol production pathway (Figure 1 A), the biomass handling involves transportation of the material to the process location and energy intensive drying to preserve the material for long time storage. Whereas in the proposed ISP method (Figure 1 B), instead of dry storage, the biomass is mixed with certain microorganisms, which in turn prevent unwanted microbial growth, and also act as a pretreatment where partial structural breakage of cellulose occurs. It is also hypothesized that the harshness of current thermo chemical pretreatment can be reduced in ISP, since the material after ISP may be softened by the microbial growth.

Major advantages of ISP are: - it doesnot need energy intensive drying step (reduces the total energy needed for the production of ethanol); less fermentation inhibitors are produced due to the reduction in severity of thermo chemical treatment, thus allowing higher dry matter content during the fermentation, and subsequently decreasing the distillation energy. The whole process becomes more environmentally friendly due to the energy and water savings.
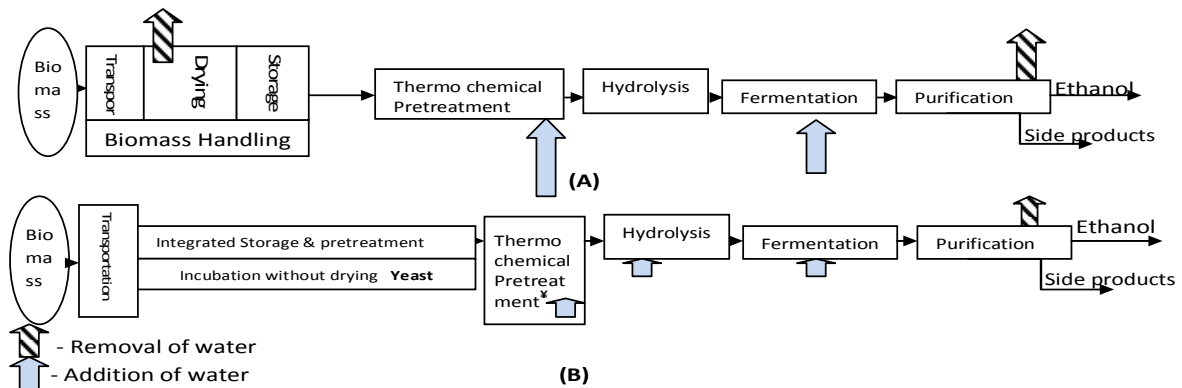
Figure 1: An ethanol process flowchart. (A)Traditional ethanol process includingbiomass handling with drying and storage. (B) Integrated storage and pretreatment without drying process. The size of arrows represents approximate quantity of water addition or removal. ¥ - Less severe thermo chemical pretreatment.

2. AIM

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

It investigates:

- Ethanol yieldsfrommoist wheat straw treated with the three fungal species *Pichia anomala, Pichia stipitis,* and *Anthracophyllum discolor* duringairtight storage in two different incubation temperatures for one month.
- The microbial characteristics during moist storage.

3. MATERIALS AND METHODS

3.1. Wheat straw

Dry wheat straw obtained from Swedish University of Agricultural Sciences (SLU) was milled (Ultra centrifugal mill ZM 1000; Retsch Germany) into fine powder and kept air tight in a plastic bag in 2°C until use. The moisture content of the wheat straw was 5.78% correspondingto a water activity of 0.223.

3.2. Water activity

Water activity was measured in Aqua Lab CX-2 (Decagon Devices inc. Washington, USA). It uses a chilled-mirror dew point technique to measure the water activity (a_w) of the sample. Dew was continually formed when the stainless steel mirror within the sample chamber is repeatedly cooled and heated. The detector calculates the relative humidity of the sample by an equilibration process [12].

3.3. Moisture content

Moisture content was measured in Sartorious Moisture analyzer MA-45 (Göttingen, Germany).It represents the amount of moisture of the sample. The moisture content of the sample includes not only the free and bound water but also all the volatile substances in the material like fats, oils, organic solvents, flavorings, product of decomposition etc. ACeramic Infrared heater was used for heating the sample to maximum of 240°C.Fully Automatic Mode was used which ends the heating cycle as soon as the weight loss per 24 second is below the automatically detected threshold[13].

3.4. Water activity adjustment

The water activity of the dry wheat straw was not adequate for the yeast cells to grow. Water activity of the intended material during storage was a_W 0.93 (from previous study[14]). Therefore the samples were adjusted to a water activity level just below the intended value byaddition of autoclaved distilled water to give room for the liquid inoculum. The samples were incubated in 2°C for 3 days for the normalization of moisture.

3.5. Media

3.5.1. Selective medium for Yeast

YPD medium (Yeast extract 10 g/L, Peptone 20 g/L from Oxoid LTD Basingstoke, New Hampshire, Glucose 20 g/L (DuchefaBiochemie B.V, Duchefa Netherlands)) was 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. The medium was sterilized by autoclaving at 121°C at 15 psi for 30 minutes, and plated in sterile conditions.

3.5.2. Selective medium for Enterobacteria

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. Enterobacterial population in a sample is an indicator of hygiene.It was used as a measure of the effectiveness of conservation[15].

3.5.3. Selective medium for Aerobic Bacteria

For the selective growth and enumeration of aerobic bacteria,Tryptone Glucose Extract Agar (TGEA) (24 g/L, Oxoid LTD Basingstoke, New Hampshire) was used. 1 ml of sample was used for the pour plate technique.

3.5.4. Liquid Growth medium

YPD without agar added was used liquid culture for yeast growth. For the liquid culture preparation of Mold during DNA isolation, YM-broth (Yeast extract 3 g/L, Malt extract 3 g/L, Bacteriological peptone 5 g/L, Glucose 10 g/L, MgSO$_4$ 7 g/L; Merck KGaA, Darmstadt, Germany) was used without any antibiotic. All the media were autoclaved at 121°C at 15 psi for 30 minutes and cooled to room temperature before use.

3.5.5. Sample extraction for Microbial counting

6 g of wheat straw was mixed with 44 ml of peptone water (Bacteriological peptone 2 g/L, Tween 80 0.20 g/L (Kebo AB Stockholm, Sweden) in a sterile polythene stomacher bag and extracted at normal speed for 180 sec in a laboratory stomacher (Seward Stomacher®; Lab Blender400C). 1 ml of this sample was serially diluted in peptone water and plated in agar plates. Distinctive colonies were counted in appropriate dilution plates and expressed as log CFU/g of wheat straw.

3.6. Fungi used in the study

Pichia anomala, a known biocontrol yeast; *Pichia stipitis*, a xylose fermenting yeast and *Anthracophyllum discolor* were used in the present investigation for storage purpose. *A. discolor,*originally isolated from decayed wood in the rainforest of southern Chile [16],is a white rot lignolytic mold. *Saccharomyces cerevisiae* was used for the fermentation process. The strains used in the study are listed in Table 1.

Table 1: Fungal strains used in the study

Sl.No	Fungal strains	Remark
1	*Pichiaanomala* J121	Biopreservative yeast.
2	*Anthracophyllum discolor*†	Lignolytic, white rotfilamentous fungus
3	*Pichiastipitis*J563 (CBS 5774)	Xylose fermenting yeast
4	*Saccharomyces cerevisiae*J672	Fermentation yeast

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

3.7. Inoculum Preparation

All yeast strains were grown on YPD master plates and stored in 2°C until use. Liquid cultures for yeast strains were prepared in YPD without agar and incubated at 25°C at 100RPM for 2 days.The cells were then centrifuged (Beckman J6-HC) at 2000 g for 10 min and washed with normal saline (9 g/L NaCl). The pellets were resuspended in normal saline and used as inoculum. *A. discolor* was originally grown on a malt extract agar master plate (Donated by Dr. Leticia Pizzul) and stored in dark at 25°C. Since scraping of mold mycelium from the agar plate was difficult, *A. discolor* was grown in two 100 ml conical flasks with YM broth at 25 °C in a static culture with occasional mixing. The mycelium was transferred in an inoculation loop to the wheat straw.

3.8. Yeast cell counting

The cells were quantified using a Hemocytometer (Scherf, Burker, Germany) under an Olympus BH2 Research Microscope (Olympus America Inc.). Approximately 100-120 cells were counted and averaged for inoculum preparation.

3.9. Optical Density (OD)

Optical density was measured in an Ultrospec 1100 Pro, Biochrom (Agilent, Germany) spectro photometer. Normal saline (9 g/L NaCl) was used as blank for the inoculum OD measurement. The cell suspension was diluted or concentrated by centrifugation and re-suspending in normal saline to get an OD value between 0.1-0.4 for accurate reading. The wave length used was 600nm.

3.10. Storage and Experimental Setup

The water activity adjusted wheat straw was separated into groups (for different species) and then liquid inoculum was added into a blender (Electrolux) and mixed thoroughly. The inoculated wheat straw was filled into labeled 50 ml Falcon tubes. Four parallels for each species were stored in 4°C and another set at 15°C for one month. The caps of the falcon tubes were kept loosely tightened to simulate air leakage during storage.

3.11. Dilute Acid Pretreatment

Dilute acid pretreatment was done in 100 ml serum flasks with aluminum foil as cap. The procedure has been described in details in my previous work [14]. The wheat straw after storage was carefully transferred into serum flasksto get a final concentration of 78.3 g/L with 0.75 % H_2SO_4. Moisture content of the dry sample was adjusted with distilled water just before the acid addition to get a same treatment for the entire set of samples. After the addition of H_2SO_4,the bottles are autoclaved in a special autoclave Uniclave 360 (Sjukhusservice AB, Vemdalem,Sweden) for 1 hr 30 min (20 min to achieve the temperature and pressure; 30 min holding and 40 min cooling. After 40 min cooling pressure was released manually.

3.12.   Simultaneous saccharification and fermentation (SSF)

After acid pretreatment pH of the sample was measured using a pH meter (PHM 92 Lab pH Meter, Radiometer; Copenhagen). The hydrolysate was highly acidic (Aprox pH 1.6-1.8), whereas pH 5 was the optimum for the cellulase enzyme to perform. pH of the solution was carefully adjusted to pH 5 using6.25 M NaOH. Since the wheat straw may contain some slow titrating substance, a known volume of alkali (~5 ml) was added to all the bottles and allowed to stand for half an hour with frequent mixing. Extreme care was taken to avoid any contamination. 70% ethanol was used to sterilize the pH electrode.

After the pH adjustment, Accellerase^{TM} 1000 enzyme (Genencor, Copenhagen) was added at a concentration of 0.2 g/g of dry wheat straw. *Saccharomyces cerevisiae* was added at a final optical density of one. The concentration was adjusted to 60 g/L by adding autoclaved distilled water. A rubber stopper was used to create perfect air tight situation. Atmospheric air was flushed out by nitrogen gas using two needle puncture on the cap. A needle (0.40X40mm/27GX 1$^{1/2}$) (Braun Sterican Disposable Needles; North Yorkshire, United Kingdom) loosely covered with an aluminum foil was used for the occasional release of carbon dioxide. The bottle was incubated in a shaker at 140 RPM at 40°C for 5 days. Samples were withdrawn using a sterile needle through the rubber stopper after 3 and after 5 days of fermentation.

3.13.   Sugar and Ethanol analysis

3.13.1. High performance liquid chromatography (RIDdetector)

Glucose, ethanol and acetate were measured using High performance liquid chromatography with refractive index detector in an Agilent 1100 series (Agilent Technologies; Waldbronn, Germany). HC-75 (Skandinaviska Genetec AB, Sweden) column was used. A 30 minute elution time was used with 0.005 M H_2SO_4 as eluent at a flow rate of 0.06 ml/min. The column was kept at 60°C. Areas under the peakswere quantified by comparison with standard solutions of concentrations varying from 1g/L to 30 g/L.

All the samples were filter sterilized using a 0.2µmmembrane filter (Sarstedt, AG & Co, Nümbercht; Germany). The HPLC vials (Agilant Technologies;Waldbronn,Germany) were stored at -20°C for later analysis and centrifuged at 13000 RPM just before loading to the HPLC to precipitate any particle.

3.13.2. High Performance Anion Exchange-Pulsed amperometric detection (HPAE-PAD)

Simple sugars like xylose, mannose, arabinose and galactose were quantified using a Dionex ICS 3000 chromatogram (Dionex Sweden AB.Göteborg-421 30) using Pulsed Amperometric Detection (HPAE-PAD). The column used was a CarboPac PA10 4×250 mm/guard column 4×50 mm coupled with gold working electrode. Chromeleon 6.80 (Service Pack 4) was used for process control. 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[10].

The solvents used for elution medium were:

Solution A: 100% deionized H_2O

Solution B: 0.2 M NaOH

Solution C: 0.2 M NaOH + 0.5 M NaOAc

Table 2: Program used for theHPAE-PAD HPLC system.

Time	Solution A	Solution B	Solution C	Remark
12 min to -7 min	0%	94.2%	5.8%	Regeneration of column (isocratic elution)
-7 min to -6 min	100%	0%	0%	Rapid gradient
-6 min to 0	100%	0%	0%	Preparation before separation (isocratic elution)
0Min			Sample injection	
0 to 17.5 min	100% A	0%	0%	Separation 1: elution of most monosaccharides
17.5 min to 29 min	50 to 1%	1 to 50%	50%	Separation 2, elution of some monosaccharides and some disaccharides
29 min to 35 min	0%	94.2	5.8%	Separation 3: elution of some Oligosaccharides

3.14. DNA Fingerprinting

3.14.1. Colony PCR for Yeast

A fresh colony of yeast was picked with a steriletooth pick and dissolved in 200 µl of 50 g/L NaOH in a PCR tube. The cells were lysed in a thermo cycler at 95 °C for 10 min. The lysed cell suspension was centrifuged in a tabletop centrifuge for two min. Tubes were kept on ice until further use.2 µL of the supernatant was used for each PCR.The (GTG) $_5$forward-reverse primer was used for all DNA finger printings(Table 1and 3.16).

3.14.2. Colony PCR for Bacteria

A fresh individual colony of bacteria was picked using a sterile tooth pick and dissolved in 200 µl of autoclaved distilled water in a PCR tube. The cells were lysed in a thermo cycler at 95 °C for 5 min. It was then centrifuged at maximum speed in a tabletop centrifuge for 2 minutes. Tubes were kept on ice until further use.1 µL of the supernatant was used for each PCR. (GTG) $_5$ forward-reverse primer was used for all DNA finger printings (Table 3and 3.16).

3.15.   DNA isolation

3.15.1. Filamentous Fungi (Molds)

The mold DNA was isolated according to Cenis 1992[17], with some modifications. Molds were grown in 15 ml falcon tubes suspended in 7 ml of YPD medium (No antibiotics were used). The cultures were incubated in 25°C for 5 days, with daily unscrewing of the cap for air addition under sterile conditions. The mycelia weretransferred to a 1.5 ml eppendorf tube with a sterile forceps. 350 ml of extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) were added and the mycelium was crushed with a conical grinder using a motorized cell agitator for 1 minute. 150 µl of 3 M sodium acetate (ph 5.2) was added to the homogenized suspension and incubated at -20° C for 10 minutes. Tubes were centrifuged at 13000 RPM for 5 minutes and supernatant was transferred to a fresh tube. An equal volume of isopropanol was added and incubated for 5 minutes at room temperature. The DNA was then pelleted by centrifuging at 13000 RPM for 4 minutes. The pellet was washed with 70% ethanol and air dried on a sterile bench. The pellet was dissolved in 50µl of TE (10 mM Tris pH 8, 1 mM EDTA)buffer and stored in -20°C.

3.16.   Polymerase Chain Reaction (PCR)

Illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare UK Limited;Little Chalfont, Buckinghamshire)were used for all reactions. The master mix was prepared according to the manufactures direction. PCR were carried out in aBioradC1000™ Thermal Cycler (Bio-Rad Laboratories; Atlanta, Georgia, USA).

Table 3: Primer used in the study

Sl.NO	Name	Sequence	References
1	ITS1	TCCGTAGGTGAACCTGCGG	[18]
2	ITS4	TCCTCCGCTTATTGATATGC	[18]
3	NL1	GCATATCAATAAGCGGAGGAAAAG	[19]
4	NL4	GGTCCGTGTTTCAAGACGG	[19]
5	16SSf	AGAGTTTGATCCTGGCTC	[20]
6	16SSr	CGGGAACGTATTCACCG	[20]
7	(GTG)$_5$	GTG GTG GTG GTG GTG	[21]

3.16.1. rDNA amplification

The D1/D2 region of yeasts 26 rDNA was amplified using NL1 and NL4 primers (*Taq* Gold polymerase; Applied Biosystems, Foster City, CA). The PCR conditions were 95°C for initial denaturation. 32 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min; and a final extension of 72°C for 3 minutes.

Internally transcribed spacer (ITS) (a piece of non-functional RNA situated between structural ribosomal RNAs on a common precursor transcript; due to the high copy number in the genome, it is easy to amplify and has a high degree of variation even between closely related species) of filamentous fungi were amplified using ITS1 and ITS4 primers (Table 3). An initial denaturation of 95°C for 3 minute, followed by 32 cycles of 95°C for 30 sec, 53°C for 30 sec, 72°C for 1 min; and a final extension of 72°C for 3 minutes were used for mold.

16S region of enterobacteria was amplified using 16SSf and 16SSr primers (Table 3). An initial denaturation of 95°C for 2 minute, followed by 29 cycles of 94°C for 30 sec, 49°C for 60 sec, 72°C for 1 min; and a final extension of 72°C for 3 minutes were used for bacteria.

3.16.2. PCR Product Purification

All the PCR products obtained from molds were loaded into a 1% agarose (UltraPure™ Agarose; Invitrogen Ltd ,Paisley PA4 9RF, UK) gel, mixed with 3 µl of 6X DNA loading dye (Fermentas AB; Sweden) and electrophoreses (Thermo Scientific Owl B2 EasyCast Mini Gel System; Thermo Fisher Scientific Inc) were done at 110 V/cm, 80mA for 1 hr. Distinctive bands were cut from agarose and a gel-purification procedure was done using QiaQuick™ Gel extraction kit(Qiagen, Germany).

4 µl of all yeast amplified products and some of the bacterial amplification products were used for electrophoresis and bands were visualized under UV trans illuminator. Remaining PCR products were purified using PCR-product purification step according to the protocol of QiaQuick™ PCR- purification kit.

3.17.   Sequencing

20µl of purified PCR products were numbered and send for DNA-sequencing facility at Macrogen sequencing service (Macrogen Corp. Europe, Amsterdam; The Netherlands) along with separate vials of respective PCR primers. A BLAST search was conducted to find out the match with the resulting sequence using NCBI BLAST service (http://blast.ncbi.nlm.nih.gov)

3.18.   Statistical analysis

All statistical tests were performed by using SAS 9.2 (SAS Institute, 2008 - USA). Significant effects between the different treatments were evaluated by one-way ANOVA followed by Tukey multiple comparison test. Comparisons were deemed statistically significant when $p \leq 0.05$.

4. RESULTS

4.1. Moisture content and water activity analysis

Moisture content of the dry wheat straw was 5.72 %, corresponding to water activity of 0.234 a_W. Water activity is a measure of the amount of free water available to the living organisms in a sample.This water activity of dry wheat straw was not adequate for yeast growth. So the water activity was increased to0.93 a_W[14]by addition of distilled water to support the yeast growth.Moisture content of the sample after water activity adjustment was 24.8% (75.2 % dry matter).

4.2. Microbial quantification

The samples after storage were analyzed for microbial cell count (Figure 2). *P. stipitis* inoculated wheat straw incubated at 15°Cshowed the highest fungal growth (8.63 X 10^7 CFU/g of wheat straw) compared to all other samples. At the same time aerobic bacterial growth was also highest (7.5 X 10^5 CFU/g of wheat straw) for the same. *P. anomala* inoculated wheat straw (4.75X 10^7CFU/g of wheat straw) showed the highest fungal colony counts among all other inoculated wheat straw samples in low temperature. *A. discolor* inoculated wheat straw at higher temperature showed the least microbial growth (each10^4Enterobacteria and Fungi and 10^6 aerobic bacteria CFU/g of wheat straw) at both storage temperatures. According to Swedish board of statues, enterobacterial load of feed sample should be below 10^6 CFU/g [22]. In all the storage situations, the enterobacteria load was far below 10^6 CFU/g of wheat straw, which indicates a goodhygiene of the storage.
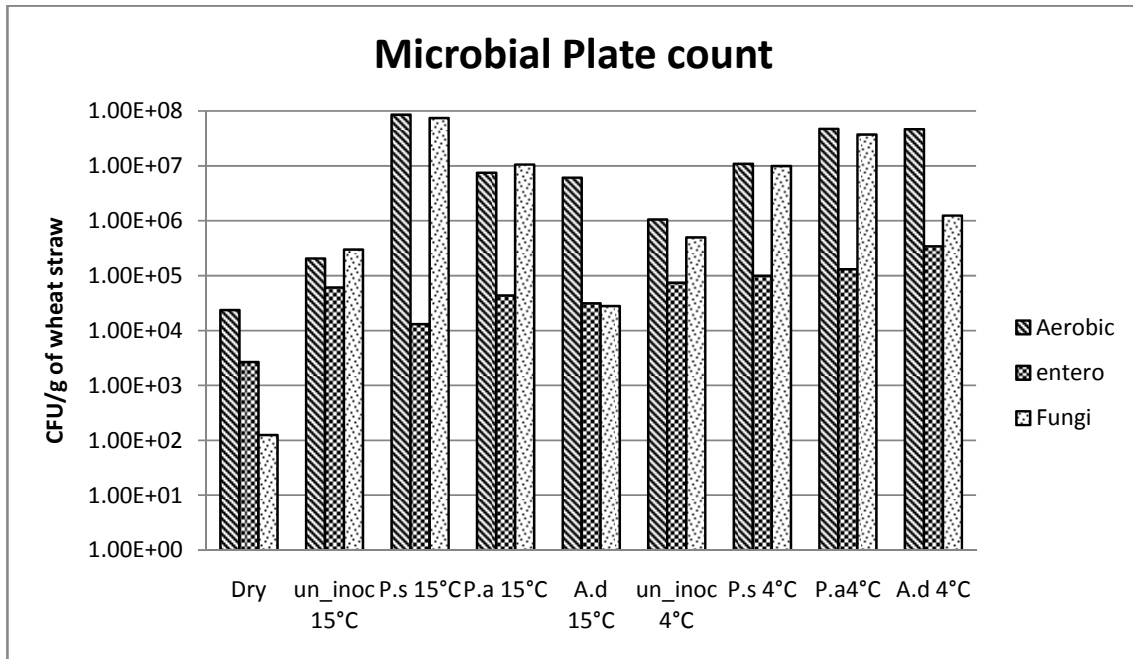

Figure 2: Microbial quantification on wheat straw after one month of storage. (Dry- dry wheat straw (Control), Un_inoc- uninoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, P.s- *P. stipitis* inoculated wheat straw. A.d- *A. discolor* inoculated wheat straw.15°C and 4°C represent the respective storage temperatures.)

It is interesting to note the ratio between aerobic bacterial CFU / fungal CFU during storage (Table 4). The ratio was approximately 1 for the samples uninoculated wheat straw at 15°C, *P.stipitis* at 15°C *P. anomala* 15°C, *P.stipitis* at 4°C, *P. anomala* at 4°C whereas the ratio was much higher than one for the dry wheat straw sample, *A. discolor* incubated at both temperatures, and uninoculated wheat straw incubated at 4°C. It is logic to assume an inert state (spore) of growth in dry sample, which caused the high ratio (190). The ratio was very high (218) for the *A. discolor* inoculated sample which showed the least fungal growth (Figure 2). This may suggest that, microorganisms are in an inert state in those samples where the ratio between the between aerobic bacterial and fungal CFU were higher than 1. Nutrients in the sample may have completely utilized and the microorganisms were moving to a dormant state (death phase or spore forming) in those sample. Also it was shown previously [14] that during wet storage, the microbial growth increases during 2 weeks of incubation time and then decreases.Even though these data suggests a microbial degradation in those samples, more experiments (plating the spores alone in microbial plate) have to be performed to prove this assumption.

Table 4: Ratio between Aerobic bacterial and fungal CFU during storage.(Dry- dry wheat straw (Control), Un_inoc- no inoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, P.s- *P. stipitis* inoculated wheat straw. A.d- *A. discolor* inoculated wheat straw. 15°C and 4°C represent the respective storage temperatures.)

Samples	Aerobic bacteria/Fungi	Ratio-Approximation
Dry	190.00	190
un_inoc 15°C	0.69	1
P.s 15°C	1.15	1
P.a 15°C	0.71	1
A.d 15°C	217.78	218
un_inoc 4°C	2.13	2
P.s 4°C	1.10	1
P.a4°C	1.27	1
A.d 4°C	37.50	38

4.3. Biocontrol activity

It is logic to assume a higher microbial growth in any sample where moisture is more present. So the microbial load of moist wheat straw was taken as the reference to biocontrol activity. More over microbial growth will be retarded at low temperature. So a particular microorganism (in this case, the 3 inoculated fungal species *P. anomala*, *P. stipitis* and *A. discolor*) have a biocontrol activity if the microbial load of a particular sample is less than moist sample(here uninoculated wheat straw) at the same temperature. So a graph was plotted (Figure 3) with microbial load for the samples with reference to the moist uninoculated wheat straw at the respective temperature.
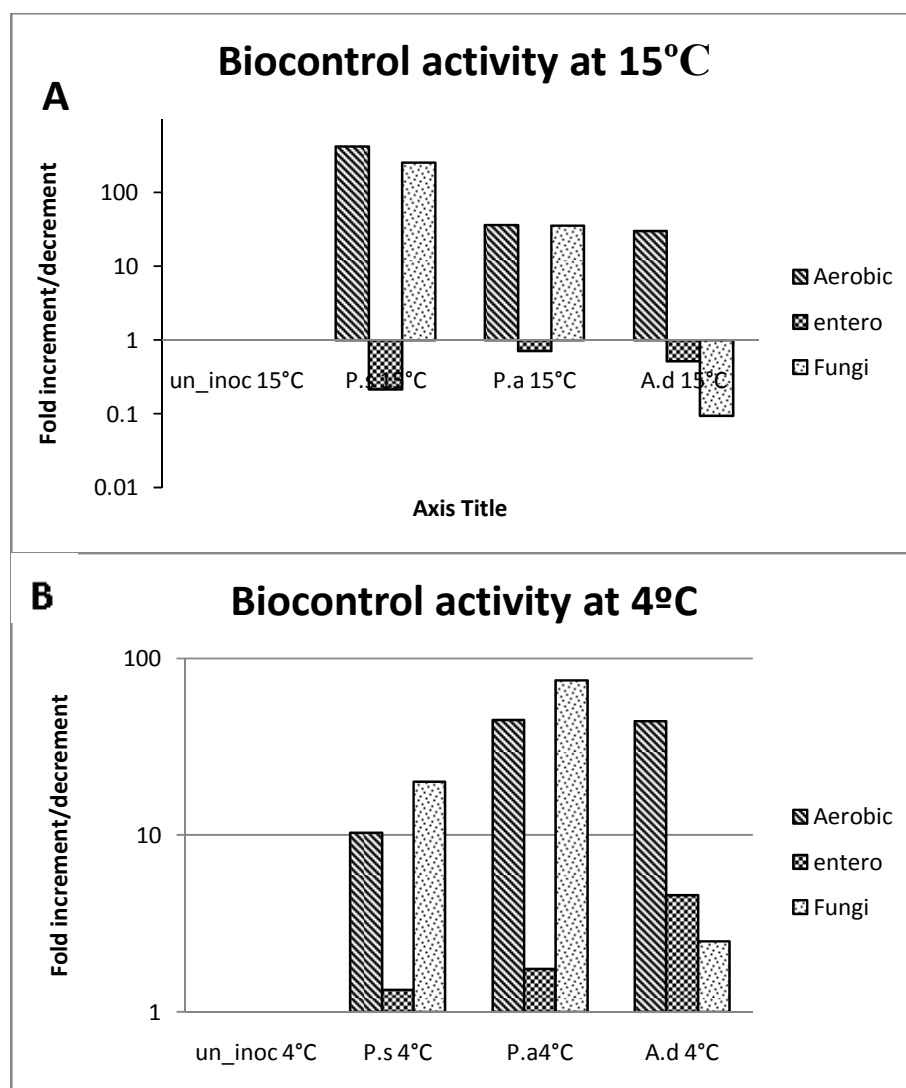
Figure 3: Biocontrol activity. A- Microbial growth with reference to microbial load of uninoculated wheat straw incubated at 15°C. B- Microbial growth with reference to microbial load of uninoculated wheat straw incubated at 4°C (Dry- dry wheat straw (Control), Un_inoc- no inoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, P.s- *P. stipitis* inoculated wheat straw. A.d- *A. discolor* inoculated wheat straw. 15°C and 4°C represent the respective storage temperatures.)

From the graph (Figure 3 A) it is clear that there isdecrease in microbial load for enterobacteria population for all the inoculated samples at 15°C. Also there is a reduction in both enterobacterial population and yeast population for *A. discolor* inoculated sample. It might be due to lack of nutrient degradation in those samples. In all the yeast inoculated samples the fungal population was a monoculture of the respective yeast. That is *P. anomala* and *P. stipitis* were totally inhibiting all other molds and yeasts in those samples.

Whereas, no distinctive biocontrol activity was observed in samples incubated at 4°C (Figure 3 B). But when looking into the microbial identification (**Table 5**), one out of the fiveVRBG

21

colonies was identified as *Acremonium strictum*, which is a yeast. In the samples inoculated with *P.stipitis*, two out of the five colonies were identified as a bile tolerant strain of *P.anomala*. Similarly, all the enterobacterial colonies from *A.discolor* inoculated sample turned to be mold, that implies that some of the counted enterobacteria in those samples represents some yeast and mold too. In short, the enterobacteria population might be less than the number represented in the graph.

Since it was clear from the study that, the enterobacteria population was always well below the hygiene standard and the aerobic bacteria might be producing spores, and the actual number might be less than the counted number, the major concern turned to mold growth during storage. Mold growth in storage is an unwanted characteristic, since it causes allergies, low ethanol yield, and may promote potentially pathogenic microorganisms. A clearly visible suppression of mold colonies was observed(Figure 4) in *P. anomala* inoculated sample and *P. stipitis* inoculated samples at both incubation temperatures. The microbial plates were covered with mold colonies for uninoculated wheat straw samples, whereas the *P. stipitis* inoculated colonies were all yeast colonies with uniform colony morphology. The colonies were analyzed by light microscopy, and the grouping to yeasts or molds was confirmed.
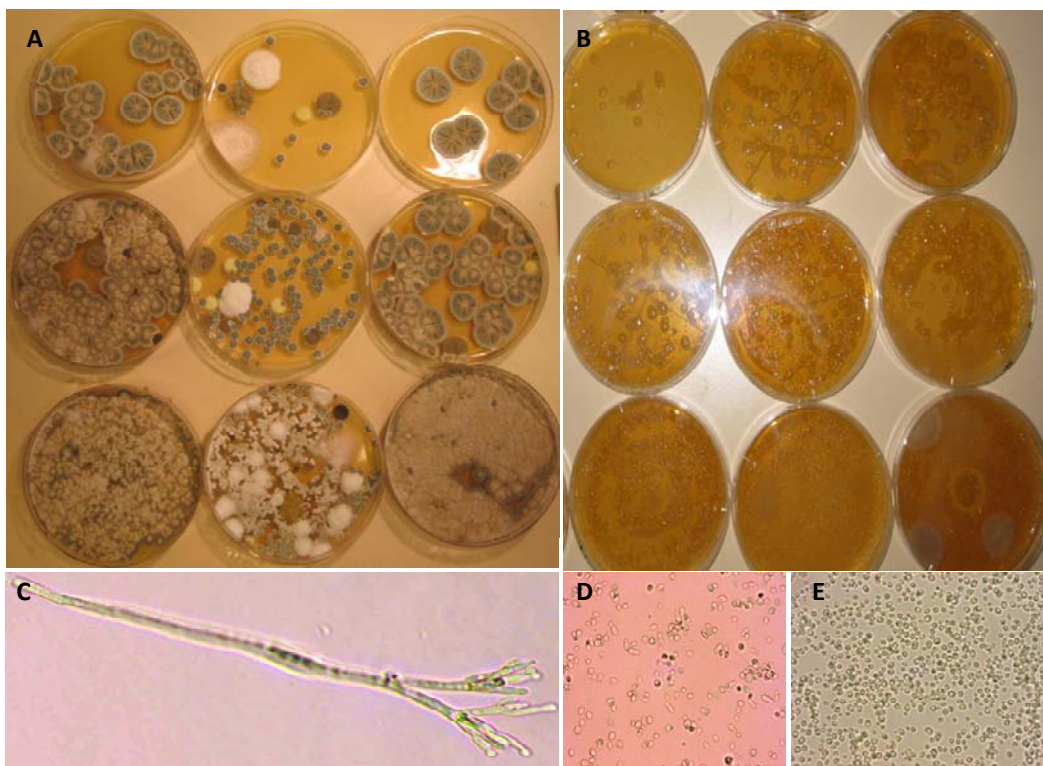
Figure 4: Inhibition of Mold by *P. stipitis*. A- Mold colonies in different dilution plates of uninoculated wheat straw sample stored at 15°C for one month. B- Yeast colonies in *P. stipitis* inoculated wheat straw samples. C- Mycelium with spore forming structure of a mold. D, E Individual yeast cells analyzed under microscope.

In conclusion, *P. anomala* and *P. stipitis* showed clear suppression of mold and other fungi as well as enterobacteria was observed during wet storage of wheat straw at 15°C. A similar activity was suspected for yeast inoculated samples incubated at 4°C, but the results were masked due to mold interference during microbial counting.

4.4. Microbial Identification

Five random (Four corners and one middle) colonies from YPD and VRBG plates were re-streaked into new plates for identification. Since VRBG was a pour plating method, the agar was pierced using a sterile tooth Pick and streaked on a new plate. Colonies were then analyzed by Light microscopy to determine whether they were formed by yeasts, bacteria or molds. Surprisingly, some of the colonies from the VRBG plates turned to be molds and yeasts. Since the colonies were tiny patches just like other bacterial colonies in the VRBG pour plates, they contributed to the number of enterobacteria during colony counting. The results were surprising and need more scrutiny, since the number of enterobacteria population was a measure of the hygiene of storage. It also explains why the higher numbers of microorganisms were observed in the samples where high fungal growth was seen. Some of the fungi were bile tolerant and able to grow in VRBG plates and counted as enterobacteria, but in fact some of them were molds.

4.5. PCR Fingerprinting

A PCR Fingerprint (Figure 5) was done for all the yeasts and bacteria to find the genetic variation and to group the same species.
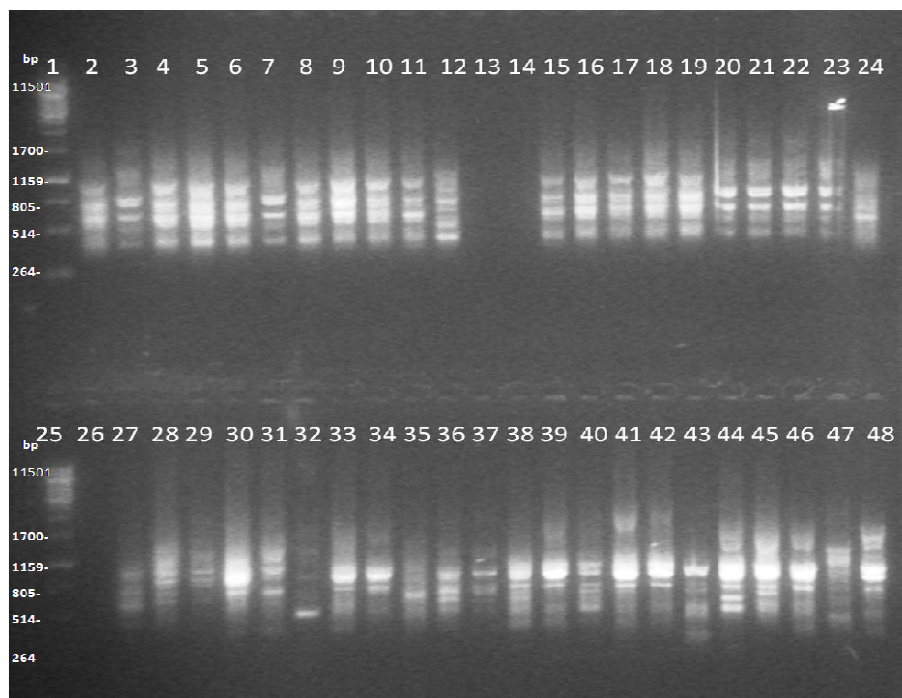

Figure 5: Example of PCR Fingerprint. Lanes 2 to 27 shows the fingerprint of Yeasts and Lanes 28 to 48 belongs to Enterobacteria Fingerprints. Lanes 1 and 25 is 1 Kb DNA ladder (Gene Ruler™ DNA Ladders – Fermentas).

Similar banding patterns were grouped together and at least one representative from each group was selected for sequencing.

The following groups were found:

Group I: Lanes2, 4, 5, 6, 8,9,10,11,15,16,17,18,19 represent*P. anomala* from master plate andyeast colonies from YPD plates of sample number 11,12,14,15,31,32,34,35,36,37, 38 respectively (see Table 5).

Group II: Lanes 3, 7, 20, 21, 22, represent*P. stipitis* from master plate, and yeast colonies from YPD plates of sample number 16,17,18,19 respectively.

12 and 24 (yeast 6 and 21) showed distinct banding, but no similarities and Lanes 13, 14and 26 (yeast from YPD plate sample number 21,22 yeast from VRBG plate sample number 34)did not show any banding pattern andwere selected for DNA sequencing. All the bacteria showed clear banding pattern, however the banding pattern of each bacteriumwas differing form each other(not all results are shown).So it was decided to select the entire set of bacterial samples for the identification.

4.6. Identification

A Morphological Identification was done to group the molds.One representative from each group was sent for sequencing. Table 5 shows the identified species with their original sample name. Some molds that were morphologically identified to the genus level are also included in the table.

Table 5: Microbial Identification. Sample 6 to 25 are colonies from wheat straw incubated at 15°C. Sample 26 to 45 are colonies from wheat straw incubated at 4°C.

Sample		Colonies from YPD Plates		Colonies from VRBG Plates	
		Organism	**Species**	**Organism**	**Species**
1	Dry1	Mold	Unidentified *Penicillium* species [P]	Bacteria	Seq Error[£]
2	Dry2	Mold	Unidentified *Penicillium* species[P]	Bacteria	Seq Error[£]
3	Dry3	Mold	Unidentified [P]	Bacteria	Seq Error[£]
4	Dry4	Mold	Unidentified [P]	Bacteria	Seq Error
5	Dry5	Mold	Unidentified [P]	Bacteria	Seq Error[£]
6	Un Inoculated	Yeast	*Aureobasidium pullulans*	Mold	Unidentified [P]
7	Un Inoculated	Yeast	*Aureobasidium pullulans*	Mold	Unidentified [P]
8	Un Inoculated	Yeast	*Cryptococcus carnescens*	Mold	Unidentified [P]
9	Un Inoculated	Mold	Seq Error[£]	Mold	Unidentified [P]
10	Un Inoculated	Mold	Seq Error[£]	Mold	Unidentified [P]
11	*P. anomala*	Yeast	*P. anomala*	Bacteria	Seq Error[£]
12	*P. anomala*	Yeast	*P. anomala*	Bacteria	Seq Error[£]

13	*P. anomala*	Mold	Seq Error[£]	Bacteria	Seq Error[£]
14	*P. anomala*	Yeast	*P. anomala*	Bacteria	Seq Error[£]
15	*P. anomala*	Yeast	*P. anomala*	Bacteria	Seq Error[£]
16	*P. stipitis*	Yeast	*P. stipitis*	Bacteria	Seq Error[£]
17	*P. stipitis*	Yeast	*P. stipitis*	Bacteria	Seq Error[£]
18	*P. stipitis*	Yeast	*P. stipitis*	Bacteria	Seq Error[£]
19	*P. stipitis*	Yeast	*P. stipitis*	Bacteria	Seq Error[£]
20	*P. stipitis*	Mold	Seq Error	Bacteria	Seq Error[£]
21	*A. discolor*	Yeast	*Cryptococcus cerealis*	Bacteria	Seq Error[£]
22	*A. discolor*	Yeast	*Cryptococcus cerealis*	Bacteria	Seq Error[£]
23	*A. discolor*	Yeast	*Cryptococcus cerealis*	Bacteria	Seq Error[£]
24	*A. discolor*	Mold	Seq Error	Bacteria	Seq Error[£]
25	*A. discolor*	Mold	Seq Error	Bacteria	Seq Error[£]
26	*Un Inoculated*	Mold	*Aureobasidium pullulans*	Mold 26	DNA
27	Un Inoculated	Mold	Unidentified [ϸ]	Mold 26	DNA
28	Un Inoculated	Mold	Unidentified [ϸ]	Mold 26	DNA
29	Un Inoculated	Mold	Uncultured fungus clone Unisequence#39-3387_3519 Φ	Mold 26	DNA
30	Un Inoculated	Mold	Unidentified	Mold 26	DNA
31	*P. anomala*	Yeast	*P. anomala*	Bacteria	Uncultured bacterium clone PP6-89 Φ
32	*P. anomala*	Yeast	*P. anomala*	Bacteria	Seq Error[£]
33	*P. anomala*	Mold	Unidentified [ϸ]	Bacteria	Seq Error[£]
34	*P. anomala*	Yeast	*P. anomala*	Yeast	*Acremonium strictum*
35	*P. anomala*	Yeast	*P. anomala*	Bacteria	Seq Error
36	*P. stipitis*	Yeast	*P. anomala*	Yeast	*P. anomala* strain CTSP F5
37	*P. stipitis*	Yeast	*P. anomala*	Bacteria	Seq Error[£]
38	*P. stipitis*	Yeast	*P. anomala*	Bacteria	Seq Error[£]

39	*P. stipitis*	Mold	Unidentified [ɸ]	Yeast	*P. anomala*IMAU:Y1036
40	*P. stipitis*	Yeast	*P. stipitis*	Bacteria	Seq Error[£]
41	*A. discolor*	Mold 41	Unidentified [ɸ]	Mold 41	Mold 41
42	*A. discolor*	Mold	Unidentified [ɸ]	Mold 41	Mold 41
43	*A. discolor*	Mold	Unidentified [ɸ]	Mold	Unidentified [ɸ]
44	*A. discolor*	Mold	*Mucorfragilis strain G6*	Mold	Unidentified [ɸ]
45	*A. discolor*	Mold	Unidentified [ɸ]	Mold	Unidentified [ɸ]

Unidentified ɸ– DNA of the samples was isolated, waiting for PCR amplification and sequencing. Seq Error-Samples does not produce any nucleotide sequence during sequencing. Φ The closest hit to any known species was below 70% sequence similarity.

One of the colonies from *P. anomala* inoculated wheat straw at 4°C was identified as *Acremonium strictum,* a potential pathogen causing diseases like mycetoma, onychomycosis (ringworm of the nail), and hyalohyphomycosis.A *Mucorracemosus* was identified (morphologically) from the TGEA plates of uninoculated wheat straw incubated at 15°C was known to cause an allergic reactions among some people.This suggests that, uncontrolled microbial growth in moist condition can result in proliferation of potential human pathogenic microorganism.

Also from the identification study, it is clear that, the *P. anomala* and *P.stipitis* inoculated wheat straw mainly contained monoculture of these species during storage. It might be due to competence, or any special suppression effect. At the same time some different strains *P. anomala* were retrieved from VRBG plates of non-inoculated wheat, might be bile tolerant, enabling survival and growth in VRBG plates.

Some of the yeast samples from *P. stipitis* inoculated wheat straw incubated at 4°Cwereidentified as *P. anomala* (Table 5: Line 36, 37, 38 and 40). The possible explanation for this switching of microorganism may be a cross contamination, or human error during sample handling. Since the samples were separated to 8 mini silos (Four silos for incubation at 15°C and other four mini silos for incubation at 4°C) after the inoculation with *P. stipitis* in a blender; one could expect the same switching of micro organism in the samples incubated at 15°C also. But all the colonies from *P. stipitis* inoculated wheat straw incubated at 15°C were *P. stipitis* itself. So the possibility of cross contamination during storage has to be ruled out. An improper labeling of microbial plates during the microbial identification might be reason for this anomaly. There was not enough time to go back to experimental steps to solve the issue since the time was limited for the thesis work

Another interesting factor was the repeated occurrence of *Cryptococcus cerealis*. The yellow slimy, comparatively fast growing yeast species might indicate a potential candidate for the ISP. The repeated occurrence proves its competence to grow in no sterile wheat straw at low temperature.

4.7. Ethanol yield

A simultaneous saccharification and fermentation process was applied to all the stored wheat straw and the dry sample for control.After the end of five days of fermentation, samples were analyzed for ethanol concentration. The ethanol values were expressed (**Figure** 6) in gram of ethanol produced from gram of wheat straw processed.

Fermentation of *P. anomala* inoculated wheat straw stored at 15°C resulted in 0.147 g of ethanol/g of wheat straw on dry weight basis (7.52 % higher than the control). *P. stipitis* incubated wheat straw at 4°C on fermentation resulted in 0.146 g of ethanol/g of wheat straw (6.87 % higher than the control). Ethanol produced from uninoculated wheat straw stored at higher temperature showed 3.9% increased yield compared to the control, whereas it showed 6.17% lower yieldsat low incubation temperature. *A.discolor* inoculated sample resulted in11.17 % and 16.62% lower yield than the control. In conclusion, out of 8 tested samples, 5 samples showed higher ethanol yields, and 3 samples showed lower yields than the control. It can be co-related to the ratio of aerobic bacteria CFU to fungal CFU (Table 4), where the ethanol yields were lower in samples where degradation was suspected during storage.
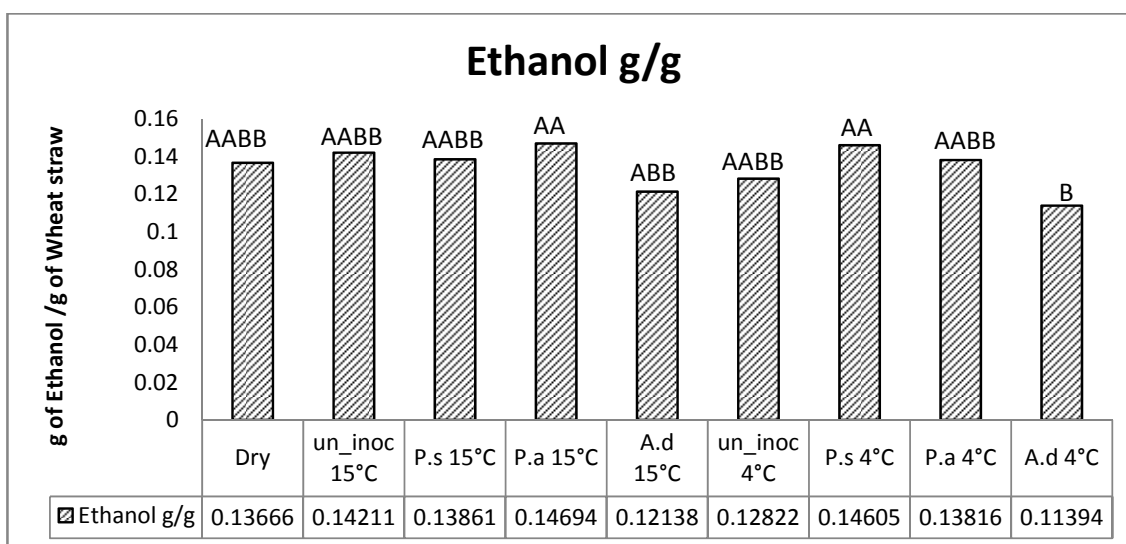

	Dry	un_inoc 15°C	P.s 15°C	P.a 15°C	A.d 15°C	un_inoc 4°C	P.s 4°C	P.a 4°C	A.d 4°C
Ethanol g/g	0.13666	0.14211	0.13861	0.14694	0.12138	0.12822	0.14605	0.13816	0.11394

Figure 6: Gram of ethanol per gram of dry wheat straw. All the values are mean of three individual biological replicates. Means with the same letter are not significantly different. (Dry- dry non stored wheat straw (Control), Un_inoc- non inoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, P.s- *P. stipitis* inoculated wheat straw. A.d- *A. discolor* inoculated wheat straw. 15°C and 4°C represent the respective storage temperatures.)

4.8. Impact of ethanol on fermentation time

Fermentation time is an important parameter in ethanol industry. Less time for fermentation implies, more substance can be processed in a given time and more products can be produced, increasing the profit. It was an assumption during the project that ISP will reduce the time of saccharification by destabilizing the wheat straw crystalline structure. Samples were analyzed for ethanol concentration in an HPLC after 3 days of fermentation (Figure **7**). From the graph, it was clear that all the ISP treated sample yielded more ethanol than the dry control except *A. discolor* inoculated wheat straw within the first 3 days of fermentation. An

explanation can be deduced from the ratio of aerobic bacterial CFU/ fungal CFU (Table 4).Samples with ratio higher than 1 showed less ethanol during the first 3 days compared to the control.That is, the easily available glucose source might have degraded away and thus resulting in lower production of ethanol for those samples. A further study with ethanol released with time (12 hrs, 24hrs, and 36hrs) might reveal this factor. In conclusion ISP has lowered the fermentation time by increasing the decrystalization of lignocellulosic material.
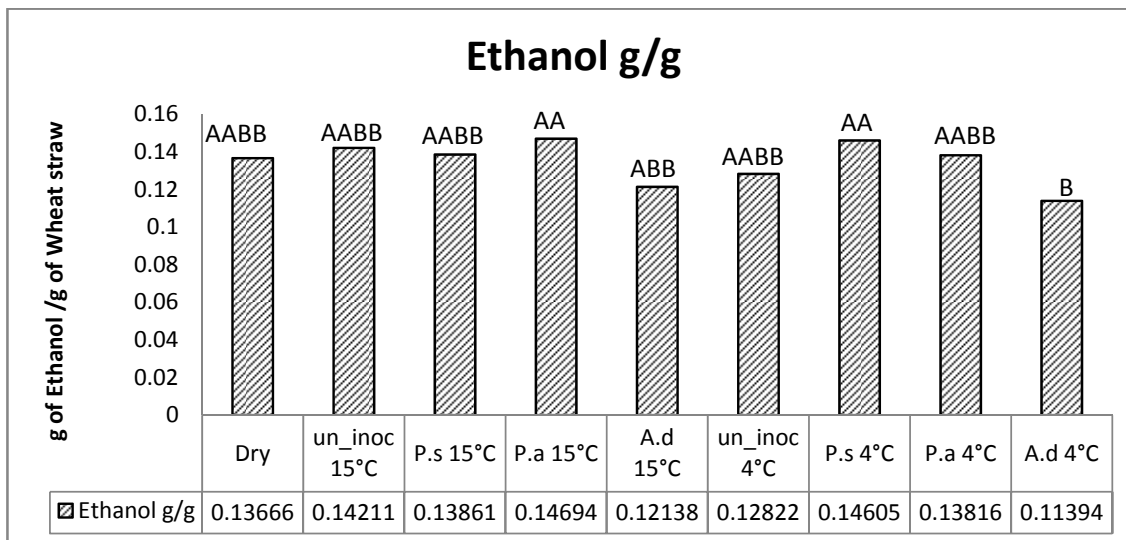
Ethanol g/g

	Dry	un_inoc 15°C	P.s 15°C	P.a 15°C	A.d 15°C	un_inoc 4°C	P.s 4°C	P.a 4°C	A.d 4°C
☑ Ethanol g/g	0.13666	0.14211	0.13861	0.14694	0.12138	0.12822	0.14605	0.13816	0.11394

Bar labels: Dry—AABB, un_inoc 15°C—AABB, P.s 15°C—AABB, P.a 15°C—AA, A.d 15°C—ABB, un_inoc 4°C—AABB, P.s 4°C—AA, P.a 4°C—AABB, A.d 4°C—B

Y-axis: g of Ethanol /g of Wheat straw

Figure 7: Ethanol release by time. All the values are means of three individual biological replicates..Et 3th Day- Ethanol released after 3 days of SSF. Et 5th Day-Ethanol released at the end of 5th day of SSF. (Dry- dry wheat straw (Control), Un_inoc- no inoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, P.s- *P. stipitis* inoculated wheat straw. A.d- *A. discolor* inoculated wheat straw. 15°C and 4°C represent the respective storage temperatures.)

4.9. Non fermented simple sugars

The Cellulase enzyme hydrolyzed the plant material and may have generated several simple sugars, some of which may not be fermentable by *S. cerevisiae*. But these sugars can be a potential source of energy for coupling different bioprocess. It is also a measure of efficiency of ISP, since more simple sugars released means more de crystallization or more pretreatment efficiency.
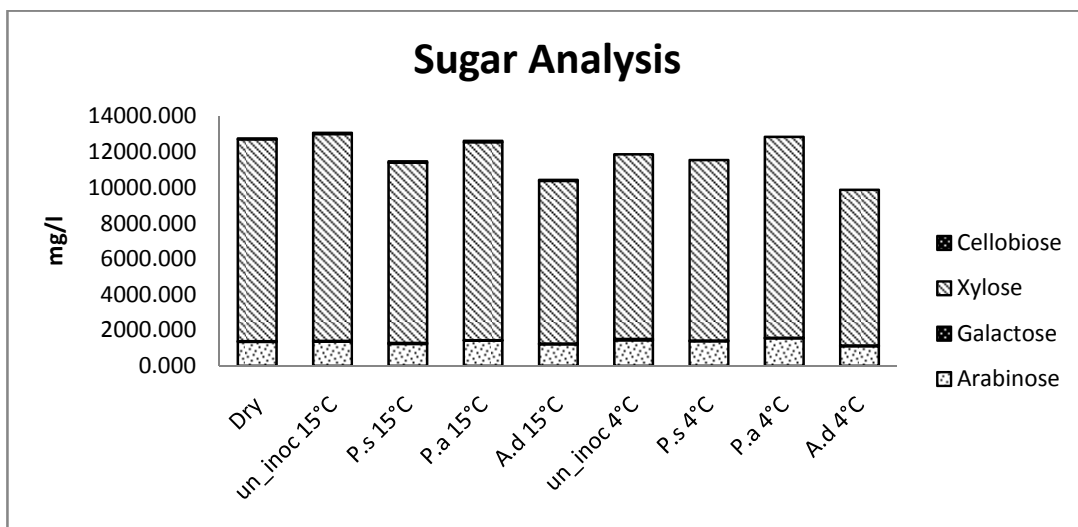
Figure 8: Sum of total non-fermented Sugar left in the medium.All the values are means of three individual biological replicates.(Dry- dry wheat straw (Control), Un_inoc- no inoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, P.s- *P. stipitis* inoculated wheat straw. A.d- *A. discolor* inoculated wheat straw. 15°C and 4°C represent the respective storage temperatures.)

Sum of total sugars released by the ISP treatment were compared to that of dry wheat straw (Figure 8). Uninoculated wheat straw at 15°C and *P.anomala* at 4°C showed more simple sugar release than that of dry control. Considering the fact that these two samples resulted in high ethanol yield, one can assume that more structural damage was created by the ISP, causing more release of sugar.
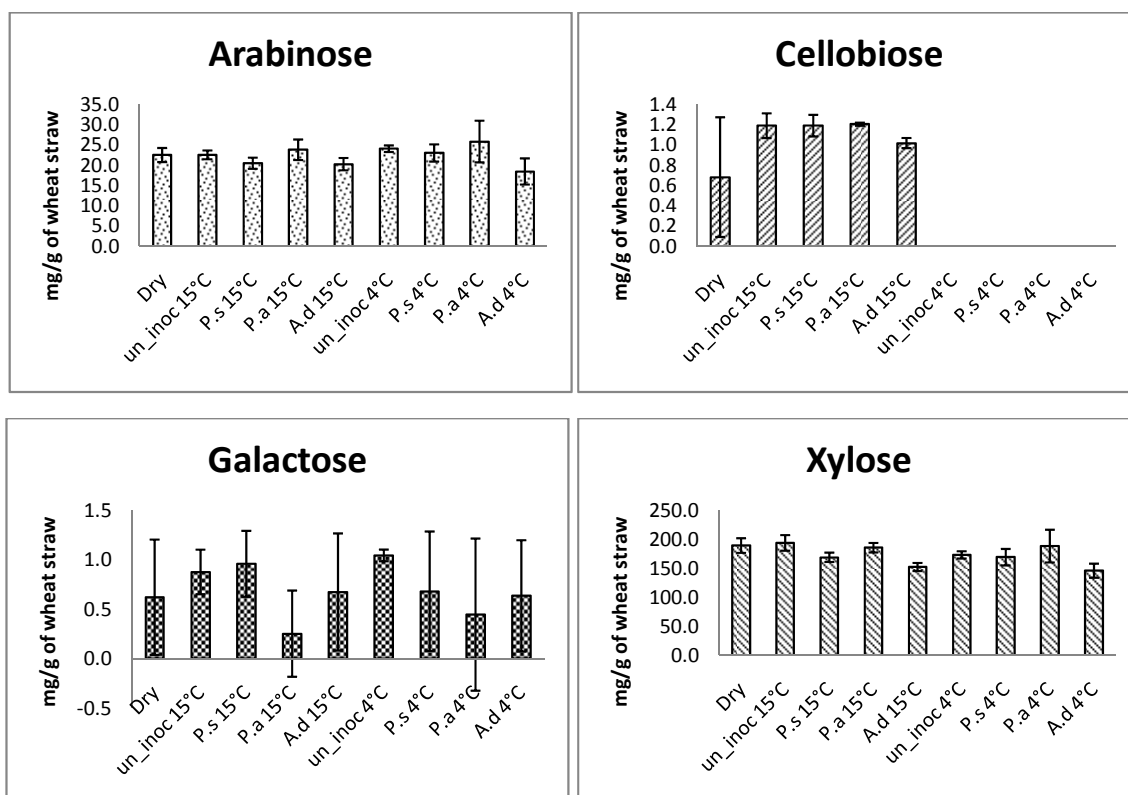
Figure 9: Simple non fermented sugars left over in the fermentation medium. All the values are means of three individual biological replicates with standard deviation as error bars. All the readings are averages of three biological replicates, with standard deviation as error bar. (Dry- dry wheat straw (Control), Un_inoc- no inoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, P.s- *P. stipitis* inoculated wheat straw.A.d- *A. discolor* inoculated wheat straw.15°C and 4°C represent the respective storage temperatures.)

A more detailed analysis of simple sugar released has to be considered, for identifying the reasons behind this deference. Wheat straw contains hemicellulose, which on saccharification releases glucose, xylose, mannose, galactose, rhamnose, and arabinose, with glucose and xylose being the major constituents. So an effective ISP will produce more of these simple sugars, if there was any hemicellulose structural breakdown happened during storage. In Figure 8 A, B and C, we can see a higher amount of arabinose, xylose, and galactose in all the samples, except *A. discolor* inoculated wheat straw and *P. stipitis* inoculated wheat straw. It reinforces the hypothesis of low ethanol production from*A. discolor*incubated sample because of wheat straw degradation. In the previous study, higher ethanol was obtained from what straw inoculated with *A. discolor*than the control, because of less growth since the quantity of inoculation was very less. On the other hand*P. Stipitis* inoculated wheat straw resulted in more ethanol, but less of these simple sugars. A possible reason for this scenario is that *P.stipitis* is a xylan degrading yeast[23]. The degraded product of the hemicellulose could have been eaten up by the yeast itself, causing a high structural damage resulting in more ethanol production, and less left over sugars.

Cellobiose is a dimeric sugar of glucose. It is a degradation product of cellulose. The fate of cellobiose in an SSF, where glucosidase enzymes are active to get cleaved into two glucose molecules and fermented by the yeast resulting in ethanol. That is all the cellobiose could

eventually converted into ethanol in this particular study. In figure 8 D, all the low temperature inoculated samples, have no cellobiose. But all the high temperature inoculated samples have some amount of cellobiose with higher amount than the control. Since the concentrations were low and the standard deviation was so high (in some cases higher than the mean), it is possible that the amounts of cellobiose and galactose were near to the detection limit.

4.10. Comparison of SSF Vs normal fermentation (SF)

In my previous study[14], wheat straw was treated with same microorganisms and ethanol was produced in a subsequent saccharification and fermentation process. Since both the process parameters and materials were the same, the results are comparable. The chart of ethanol produced in previous study (Normal fermentation) was compared with Simultaneous saccharification and fermentation (Figure 10). It is clear from the comparison chart that SSF is more efficient than normal fermentation. The possible reason for this scenario, may be that during the subsequent saccharification and fermentation process, saccharification was not complete, or there were some potential polysaccharide remains which were non soluble and got wasted away during the remaining process.But in *A. discolor* inoculated sample the difference between ethanol yield of SSF and normal fermentation was quite low. Considering the fact that from the *A. discolor* inoculated samples only little ethanol was produced compared to all other SSF samplesit can be concluded that *A. discolor* (or the other microbes in the particular sample)may actuallyhave degraded the wheat straw resulting in a lower yield. This was also true for uninoculated wheat straw incubated at low temperature.
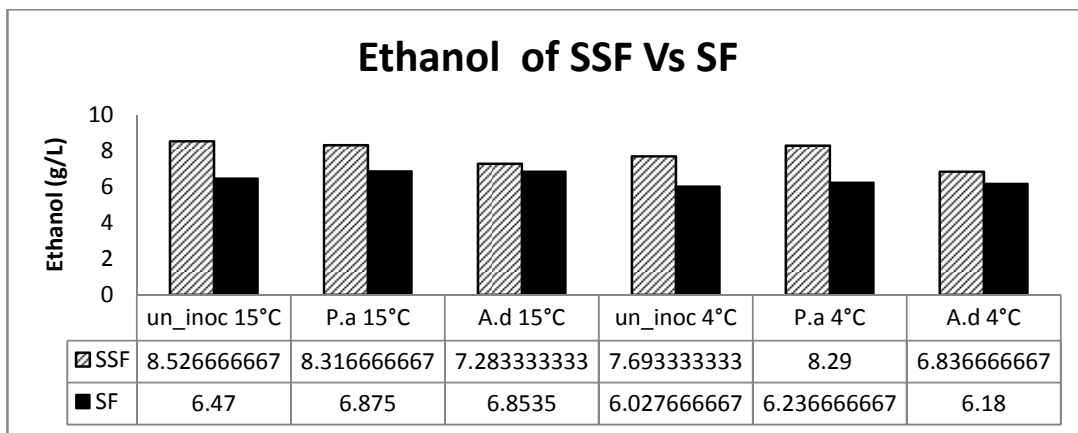

Ethanol of SSF Vs SF

	un_inoc 15°C	P.a 15°C	A.d 15°C	un_inoc 4°C	P.a 4°C	A.d 4°C
SSF	8.526666667	8.316666667	7.283333333	7.693333333	8.29	6.836666667
SF	6.47	6.875	6.8535	6.027666667	6.236666667	6.18

Figure 10:Ethanol production by the Simultaneous saccharification and fermentation (SSF)Vs saccharification and subsequent fermentation (SF). (Un_inoc- no inoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, A.d- *A. discolor*inoculated wheat straw.15°C and 4°C represent the respective storage temperatures.)

Comparison of simple sugars of SSF was done with that of SAF (Figure 11). Except uninoculated samples at 15°C, total sugars of SAF were always higher than that of SSF. Since the ultimate aim was to obtain ethanol, not to produce simple sugars, SSF can be consideredto give higher ethanol yield than saccharification and subsequent fermentation technique.
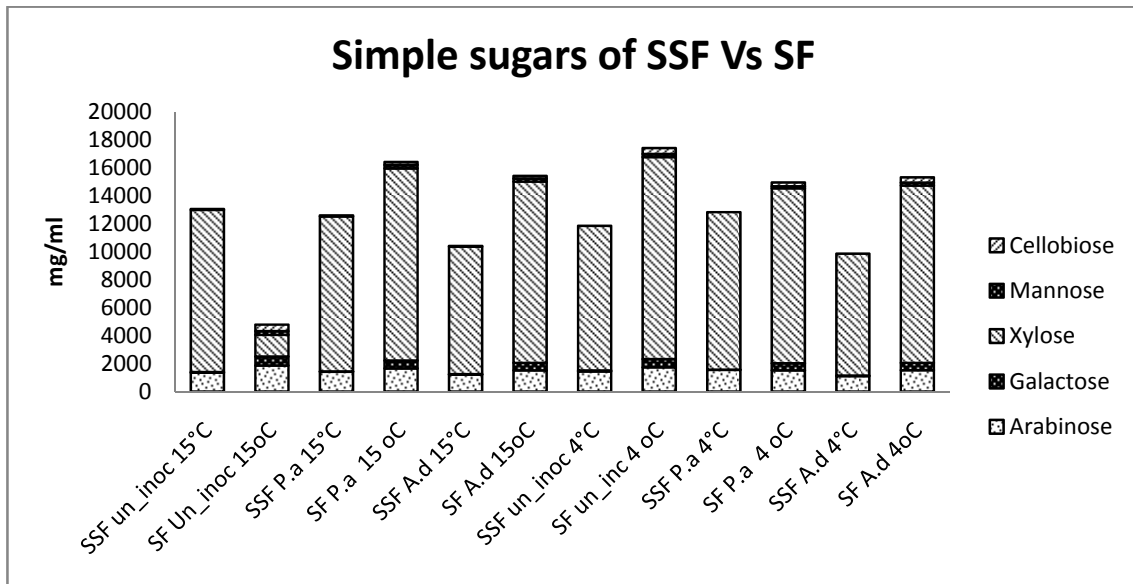
Figure 11: Comparison of simple sugars released by Simultaneous saccharification and fermentation (SSF)Vs Normal fermentation (SF). (Un_inoc- no inoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, A.d- *A. discolor*inoculated wheat straw.15°C and 4°C represent the respective storage temperatures.)

5. DISCUSSION

The ethanol yield from wheat straw treated by the ISP method was quite promising,except *A. discolo*r and none inoculated wheat straw inoculated at low temperature, whichresulted inlower yield than the dry wheat straw. But going deep into the study generates several doubts, which needs more scrutiny.

5.1. Was the choice of organism a right one?

The choice of organisms for the inoculation was an educatedguess. And it was shown that, *A. discolor* was not a good candidate for the ISP studies whereas *P. anomala* and *P. stipitis* were good candidates for ISP. But there are several other potential microorganisms, which have to be tested for this treatment. A stable microbial community has to be constructed with robust performance at any environmental conditions [7]. Even the Uninoculated sample showed a better (3.9 % higher Ethanol yields than the dry wheat straw) and faster (Figure 6, Figure 7) release of ethanol than the dry wheat straw control, indicating that the natural population on the wheat strawcontains potential candidates for the ISP. So we have to look back to the micro flora, and need to do a screening test for suitable microorganism.

5.2. Non ideal situation?

Uncontrolled microbial growth during the ISP resulted in low ethanol yield possibly due to degradation of wheat straw (refer Chapter: 4.7,4.10). It may generate a possible doubt on the delicacy of ISP that in any case if the storage time gets extended to more than one month (May due to a factory shut down or an overloaded storage) whether the microorganisms started degrading the wheat straw, and finally reduced the yield. But as it was clear from my previous study that the microbial load, reaches highest numbers after two weeks of incubation time and started to decline, the microbial load was below 10^2 CFU/g after 1 year of storage. Yet the fermentation has to be performed on long time stored wheat straw to prove the robustness of ISP.

The temperature during the entire month of storage was a constant one (either 15°C or 4°C), an ideal situation for a microbial community to colonize. Microbial colonies at one particular temperature will be different from another temperature[24], a fluctuation in temperature (A normal outdoor climate) can have a serious consequence on the ethanol yield. The study shows (4.7) a big change in ethanol yield in the same microorganism incubated at two different temperatures. For example the higher yield (1.4%) obtained from*P. Stipitis* inoculated wheat straw incubated at 15°C got even higher to 6.8% at 4°C incubation temperature; whereas the 7.5% higher yield from *P. anomala* inoculated sample incubated at 15°C got reduced to 1.09% at low temperature.So it is clear that both positive and negative effects can happen to the ISP, when the temperature of incubation varies. A stable community is needed to resolve this issue.

5.3. What is the mechanism of ISP?

The mechanism of ISP is unknown. But a correlation can be deduced from the study (refer Chapter 4.2, 4.7) that high ethanol yield will be always accompanied by a high microbial growth. But the scenario needs to be tested further, since some of the molds and yeasts were counted as enterobacteria and aerobic bacteria during the experiment. Also higher ethanol yielding ISP strains *P.stipitis* and *P. anomala* were not alone during the 1 month incubation. The Molds, bacteria and other microbes could have an impact on the structural breakdown, and release of ethanol. Revealing the mechanism of ISP will help to achieve greater yield in a more controlled manner.

5.4. SSF or SAF?

Even though SSF has several advantages like lower need of hydrolyzing enzyme, no requirement for separation, it has a major disadvantage too. SSF is the requirement for long incubation time. But it is obvious from the results (Figure 7, Figure 11) that, ISP treatment makes the release of sugar faster, resulting less time for ethanol fermentation. Alsohigher ethanol yieldsobtained by SSF compared to SAF (Figure 10) make simultaneous saccharification and fermentation more profitable than normal type of fermentation.

5.5. Thermo chemical normalization effect

Possibility of a thermo chemical normalization effect (The real difference created by the microbial growth could be masked by the intense thermo chemical treatment) cannot be ruled out by this study.Even though a literature study indicates that, theoretically Swedish wheat straw contains cellulose and hemicellulose to produce ethanol approximately 0.2 g/g of dry straw, the acid pretreatment will only result 25% of the theoretical limit (that is approximately 0.065 g of ethanol/g of wheat straw), which was far below yield obtained in the present study[25]. This suggests that the chemical pretreatment has pushed the ethanol yield to the practically possible limit of ethanol production, and to avoid this making effect we have to reduce the severity of chemical treatment. But to completely prove this assumption, a more detailed study with different pretreatment conditions has to be performed.

6. CONCLUSION AND FUTURE PROSPECTIVE

The objective of ISP is to have a controlled structural breakage of the cellulosic material, with prevention of unwanted microbial growth. Uncontrolled microbial growth resulted in degradation of the biomass and lead to a lower ethanol yield,also caused proliferation of pathogenic microorganism. Inoculation of tested yeast species was efficient in having a controlled microbial growth (decline of growth after 1 month) with a biocontrol activity (preventing other fungal and enterobacteria) and in creating a structural breakage (less severe pretreatment parameters) in the cellulosic material (higher ethanol yield).

Ethanol produced from the wheat straw, treated in ISP method with *P. anomala* incubated at 15° C for 1 month was 7.52 % higher than the dry wheat straw control.Similarlythere was an increased ethanol yield (6.8 %) for the samples treated in ISP method with *P. stipitis* incubated at4°C. Moist stored uninoculated wheat straw resulted in 1. 42 % increase, where samples with *P. anomala* at 4°C were used as biocontrol agent showed an increase of 1.09 % ethanol yield.

From the present study we can conclude a biocontrol activity for *P. anomala* and *P. stipitis* in storage of wheat straw since these samples inoculated with yeast showed suppression of mold and other fungal species. Enterobacteria inhibition was also confirmed even though the enterobacteria count was interfered with some mold colonies in low incubation temperature.

For a more effective ISP, a stable microbial community capable of higher structural breakdown without much degradation of the biomass has to be constructed. The mechanism of ISP has to be studied, and mutant microorganisms may be applied for selective degradation of biomass to get maximum structural damage without any cellulosic degradation. Thermo chemical pretreatment has to be optimized to get the maximum benefit out of microbial depolymerization. The same technique has to be tested in different cellulosic material to identify the effectiveness over common biofuel feed stocks such as wood chips and other agricultural residues. Finally the process needs to be scaled up to pilot plant study and industrial level.

7. ACKNOWLEDGMENT

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9. APPENDIX

Table 6: Microbial quantification of samples after one month storage (units are expressed in CFU/g of wheat straw).(All the values are expressed as mean ± standard deviation of three individual biological replicates. Dry- dry wheat straw (Control), Un_inoc- no inoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, P.s- *P. stipitis*inoculated wheat straw.A.d- *A. discolor* inoculated wheat straw.15°C and 4°C represent the respective storage temperatures.)

Samples	Aerobic	Entero	Fungi
Dry	2.38E+04	2.69E+03	1.25E+02
un_inoc 15°C	2.06E+05	6.13E+04	3.00E+05
P.s 15°C	8.63E+07	1.31E+04	7.50E+07
P.a 15°C	7.50E+06	4.38E+04	1.06E+07
A.d 15°C	6.13E+06	3.15E+04	2.81E+04
un_inoc 4°C	1.06E+06	7.50E+04	5.00E+05
P.s 4°C	1.10E+07	1.00E+05	1.00E+07
P.a4°C	4.75E+07	1.31E+05	3.75E+07
A.d 4°C	4.69E+07	3.44E+05	1.25E+06

Table 7: Simple sugars released. (All the values are expressed as mean ± standard deviation of three individual biological replicates.Dry- dry wheat straw (Control), Un_inoc- noninoculated moist wheat straw, P.a- *P. anomala* inoculated wheat straw, P.s- *P. stipitis*inoculated wheat straw.A.d- *A. discolor* inoculated wheat straw.15°C and 4°C represent the respective storage temperatures.)

Samples	Arabinose (mg/l)	Galactose(mg/l)	Xylose(mg/l)	Cellobiose(mg/l)
Dry	1348.70±103.98	37.15±34.94	11328.92±753.72	40.86±35.40
un_inoc 15°C	1350.82±65.27	52.48±13.48	11601.53±805.87	71.28±7.33
P.s 15°C	1226.25±82.70	57.40±19.98	10113.85±484.27	71.31±6.40
P.a 15°C	1425.85±152.32	15.07±26.11	11107.67±493.47	72.19±0.93
A.d 15°C	1213.52±91.34	40.31±35.47	9119.10±415.65	60.99±3.01
un_inoc 4°C	1441.75±48.20	62.48±3.56	10364.54±374.52	0±0
P.s 4°C	1378.46±127.14	40.7±36.20	10124.59±851.58	0±0
P.a 4°C	1547.38±308.52	26.60±46.08	11269.40±1689.68	0±0
A.d 4°C	1105.57±193.84	37.99±33.68	8732.59±731.11	0±0

Table 8: Ethanol released. (All the values are expressed as mean ± standard deviation of three individual biological replicates**.** Dry- dry wheat straw (Control), Un_inoc- noninoculated moist wheat straw*,* P.a- *P. anomala* inoculated wheat straw, P.s- *P. stipitis*inoculated wheat straw.A.d- *A. discolor* inoculated wheat straw.15°C and 4°C represent the respective storage temperatures.)

Sl No	Sample	Ethanol -3 days(g/g)	Ethanol-5days(g/g)
1	Dry	0.112±0.0025	0.136±0.0058
2	un_inoc 15°C	0.121±0.0029	0.142±0.0109
3	P.s 15°C	0.114±0.0029	0.138±0.0076
4	P.a 15°C	0.115±0.0016	0.146±0.0041
5	A.d 15°C	0.102±0.0019	0.121±0.0089
6	un_inoc 4°C	0.115±0.0067	0.128±0.0096
7	P.s 4°C	0.118±0.0041	0.146±0.0024
8	P.a 4°C	0.122±0.02169	0.138±0.0212
9	A.d 4°C	0.100±0.0098	0.113±0.0091