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

# Integrated Storage and Pretreatment of Lignocelluloses for Bio-fuel Production

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## ABSTRACT

The process for the production of lignocellulosic biofuel includes various sequential energy intensive stages like storage of biomass, thermochemical pretreatment, hydrolysis and fermentation. In the current study, an integrated storage and pretreatment method is investigated to get maximum ethanol yield in a single step process. A special 100 ml bioreactor was designed in which the thermochemical pretreatment (TCP) of wheat straw and simultaneous saccharification and fermentation (SSF) were performed. The ethanol production process was developed for wheat straw that was stored for one month at two different low temperatures with two different fungal species (*Pichia anomala* and *Pichia stipitis*) and a co-culture of both. *Pichia stipitis* inoculated wheat straw at low temperature gave 40.30% higher ethanol as compared to dry wheat straw after 96 hours SSF while a mixed culture of both species gave second highest value as compared to control. 30 minute TCP enabled higher ethanol yield (g/g) than it was obtained after 120 minute TCP, indicating less production of inhibitors during shorter TCP.

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# 1. INTRODUCTION

Global energy demand can only be met in a sustainable manner if new technological alternatives will be applied that will replace existing fossil based fuels. Worldwide energy consumption was 13.5 terawatt (TW) in 2001 by 6.1 billion people and around 86% percent of energy was derived from fossil fuels. In 2050, world population will be 9.4 billion and the calculated energy demand will be 40.8 TW. Environmental pollution has increased and that will be touching at its highest peak till 2050. This alarming situation has increased the global energy focus on alternative sources of energy (1). Bio-ethanol can be one of the possible options among other alternative sources of energy. Ethanol is mostly being produced from food items like corn grain and sugarcane that is called first generation bio-fuels but it is not sustainable due to food completion.

Ethanol can also be produced from lignocellulosic materials, like agricultural resides and forest remains. However, lignocellulosic materials are not readily fermentable due to their complex structure (2). In order to covert this complex structure a suitable process must be defined to convert it into simpler structures for easy enzyme saccharification to get fermentable sugars. Thermo-chemical pre-treatment of cellulosic biomass is the first step to break the complex structure of cellulosic biomass (3). But thermo-chemical pretreatment of lignocellulosic material, under harsh conditions, produces highly toxic compounds that inhibit the fermentation process and ultimately reduce the yield (4). In 1983, Hatakka suggested a biological pretreatment of wheat straw using white root fungi to replace thermo-chemical pretreatment. He was successful to prove that biological pretreatment can replace thermo-chemical pretreatment but his method was not feasible due to lengthy sterile incubation of wheat straw for five weeks (5). Therefore, there is a need to develop a process for the biological degradation of cellulosic biomass that would be feasible, economical and give higher ethanol yield.

Commercial ethanol production process needs storage and conservation of biomass in order to avoid unwanted microbial growth for continuous supply for ethanol production. Drying is the only method to do so but it requires high energy inputs in the countries like Sweden where the temperature is very low (6). Olstorpe and Passoth described that *Pichia anomala* has been proved as efficient biopreservative yeast by reducing the growth of unwanted mold and Enterobacteria in moist cereal grains (7). In 2009, Passoth *et al.,* showed that wheat grains gave increased ethanol yield when stored in air tight containers but no further increase in ethanol yield when stored with *P. anomala* or cellulose enzymes (8). Therefore, storage of biomass under moist conditions will not only reduce the energy for storage but may reduce the material loss by controlling unwanted microbial growth by using bio-preservative yeast. Moreover, it can also help to disintegrate the complexity of cellulosic biomass and hence a kind of pretreatment during wet storage.

Integrated storage and pretreatment (ISP) is a practical concept that is based on cellulosic biomass degradation by softening (opening) its complex structure at the time of storage. Microorganisms growing during storage do the breakage in the crystal structure of biomass partially the same way as random breakdown in thermochemical pretreatment (9).

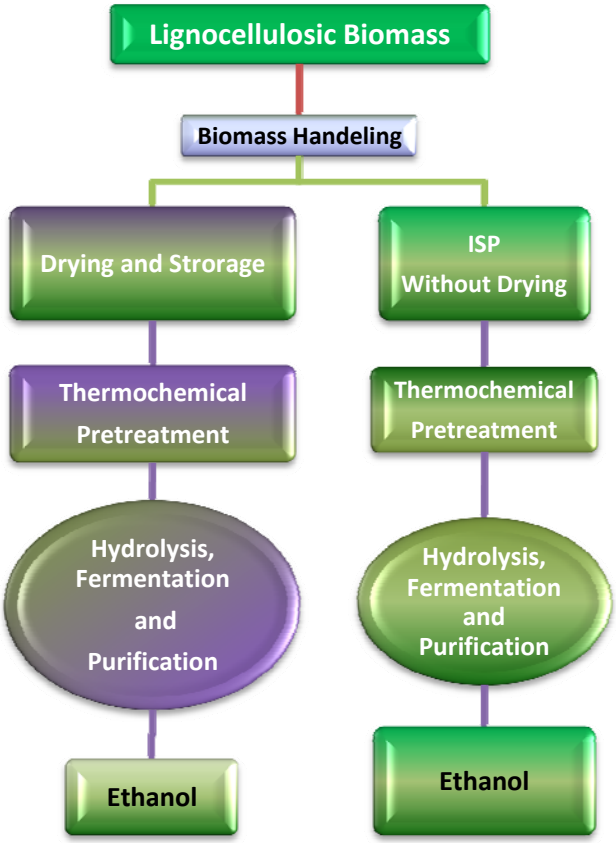



**Figure 1: Schematic Process for Ethanol Production:** Conventional method of ethanol production that includes handling of biomass with drying before storage (Left pathway) and the concept of integrated storage and pretreatment with moist storage including reduced thermo chemical pretreatment (Right Pathway), ISP shows integrated storage and pretreatment.

In the traditional process for ethanol production pathway (Figure 1, Left), biomass handling involves the energy intensive drying before storage in order to preserve the biomass for a long time while in the suggested ISP method (Figure 1, Right), wet biomass is mixed with potent microorganisms that not only soften the biomass by doing partial breakdown of biomass but also act as biopreservatives to prevent unwanted microbial growth, hence eliminating the energy intensive drying process. It can also be expected and hypothesized that ISP can reduce the thermochemical pretreatment time due to softening of biomass by microorganism during wet storage.

## 2. OBJECTIVES

The main objective of the current study was to develop an energy efficient integrated storage and pretreatment of wheat straw for high ethanol production

It investigates:

- The effect of storing moist wheat straw at low temperature with two different fungal species (*Pichia anomala* and *Pichia stipitis*) and their co-cultures on ethanol yield
- Effect of thermochemical pretreatment time on ethanol yield after ISP

# 3. MATERIALS AND METHODS

## 3.1 Sample of Wheat Straw

Sample of wheat straw used in the study was obtained from the Department of Microbiology, SLU (10) that was already in powdered form (milled into fine powder in an Ultra centrifugal mill ZM 1000, Retsch Germany). Powered dry wheat straw was stored in a plastic bag in 4°C for experimental use.

## 3.2 Measurement of Water activity

Water activity was measured in Aqua Lab CX-2 (Decagon Devices inc. Washington, USA). Water activity ($a_w$) is a measure of free water content in the wheat straw sample. It is defined as the partial vapor pressure of water in the substance divided by that of pure water at the same temperature (11). Bacteria usually require at least $a_w$ 0.91, and fungi 0.7. If water activity is kept below a certain level in the wheat straw sample, then growth of microorganism is inhibited.

## 3.3 Moisture Content

Moisture content was measured in Sartorious Moisture analyzer MA-45 (Göttingen, Germany). It represents the free water available and bound water in the sample. Calculations on every step in the current study are based on dry matter because moisture content varies from sample to sample but dry matter content remains the same (12).

## 3.4 Adjustment of water activity

The water activity of wheat straw was adjusted before storage with yeast cells because it was not adequate for the growth of yeast cells. The samples were adjusted to a water activity up to 0.973 by the addition of autoclaved water and incubated at 2°C for three days for the normalization of moisture (10).

## 3.5 Fungal strains, media and growth conditions

Three fungal species used in the study were *Pichia anomala*, *Pichia stipitis and Saccharomyces cerevisiae*. *Pichia anomala* is well known bio-control yeast (13). *Pichia stipitis* is a xylose fermenting yeast while *Saccharomyces cerevisiae* was used as fermentation yeast. 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) was used for yeast growth. Agar 2.5% (Oxoid LTD Basingstoke, New Hampshire) was used for making solid plates. All strains were grown on YPD master plates and stored in a 2°C incubator. For liquid culture *Pichia anomala*, *Pichia stipitis* were grown in YNB (Yeast Nitrogen Base) and incubated at 25°C for 24 hours at 140 rmp. *Saccharomyces cerevisiae* was incubated at 25°C at 120 rpm for overnight growth until it reached OD 1 at 600nm. The cells were centrifuged and washed with normal saline (10).

### 3.6 Yeast Cell Counting

The cells were quantified using Hemocytometer (Scherf, Burker, Germany) under an Olympus $BH_2$ Research Microscope (Olympus America Inc.) by diluting with Normal saline (0.9% Sodium Chloride Solution).

### 3.7 Optical Density (OD)

The optical density was measured in Ultrospec 1100 pro, Biochrom (Agilent, Germany) spectrophotometer. Normal saline was used as blank for OD measurements and inoculum was diluted or concentrated to get an OD range from 0.1 to 0.4 for an accurate reading at 600nm.

### 3.8 Experimental Setup for storage of wheat straw

The wheat straw samples whose water activity was adjusted to 0.973 (moisture content 30%) were mixed in blender (Electrolux) with pure cultures of *Pichia anomala*, *Pichia stipitis* and a co-culture of both ($10^8$ cells/g of dry weight of wheat straw of each). Dry and un-inoculated control samples of wet wheat straw of same water activity were also kept along with the test samples for comparison. All samples were filled in 50ml Falcon tubes and kept at 4°C and 15°C for four weeks storage. The caps of all falcons were closed tightly and a needle was introduced in each falcon to allow air leakage. There were three parallels for each sample.

### 3.9 Bioreactor design for acid treatment and SSF

In order to avoid contamination and material loss, proper mixing and oxygen removal and to obtain maximum ethanol yield special 250ml and 100 ml bioreactor were designed (as shown in figure 2) and tested in an experiment for pilot study. Controlled anaerobic conditions, thermochemical pretreatment, pH adjustments (after acid pretreatment), oxygen removal and SSF (simultaneous saccharification and fermentation) took place in the same bioreactor.

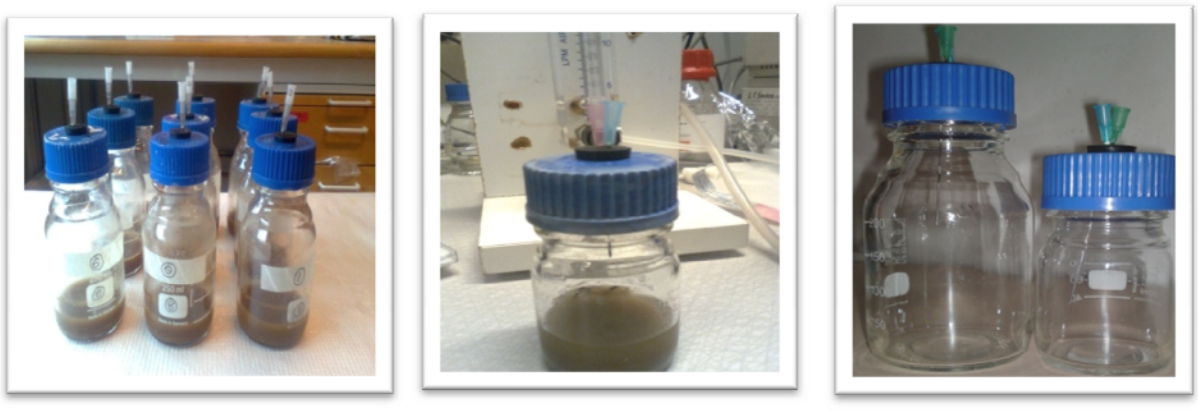


**Figure 2: Bioreactor designed for TCP and SSF:** 250ml (left), 100ml (centre) and both (right).

### 3.10 Thermo-chemical Pretreatment (TCP)

Thermochemical pretreatment (TCP) was performed in 100 ml bioreactor having very tight caps with rubber corks. A syringe needle was introduced into this bioreactor for exhaust during thermochemical pretreatment. Stored samples were carefully weighed and transferred into bioreactor and 0.75% $H_2SO_4$ was added to get 7.8g/l. Moisture of dry samples was adjusted with autoclaved water and then bioreactors containing wheat straw inoculated with yeast species were given 30 minute or 120 minute thermochemical pretreatment in an autoclave at 121°C.

### 3.11 Simultaneous saccharification and fermentation (SSF)

After dilute acid pretreatment, pH in each bioreactor was measured and adjusted to pH 5 (with 10M NaOH) solution in sterile conditions using a pH meter. Sodium Citrate buffer (0.1M) was used for keeping the pH optimum. After pH adjustments, enzyme (Accellerase DUET enzyme, Genencor, Copenhagen) was introduced into bioreactor through needle at a concentration of 0.1g/g of dry wheat straw. Accellerase DUET is a cocktail of several enzymes including, Xylanase (3600ABX/g), Cellulases (2200-2500 CMCU/g) and β-Glucosidase (400U/g). *Saccharomyces cerevisiae* cells (initial OD 1) were added into the reaction mixture and was adjusted to 8% by adding sterile water. Air from the head space in the bioreactor was removed by nitrogen flushing (flow rates varied from one liter per minute to two liters per minute) through an additional sterile syringe needle in to the bioreactor. All bioreactors were kept in a shaker at 35°C for 5 days at 120 rpm. Samples were withdrawn after every 24 hours during the SSF using a sterile needle. After taking the samples were centrifuged, filter sterilized and stored for HPLC analysis.

### 3.12 HPLC analysis

High Performance Liquid Chromatography with refractive index detector Agilent 1100 series (Agilent Technologies, Waldbronn,Germany) was used to measure ethanol, glucose and xylose concentration (10).

### 3.13. Student T-test

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

# 4. RESULTS AND DISCUSSION

## 4.1 Water Activity and Moisture Contents

The dry weight of the original wheat straw sample was 94.25 % ($0.215a_w$) corresponding to 5.75 % moisture. The water activity was adjusted 0.973, corresponding to 30% moisture content. Random analysis of moisture content of wheat straw samples were tested at the time of storage and after storage and it was observed that sample stored at 15°C showed 1 .5 to 2.0 % dry weight loss while samples at 4°C had slight change in moisture content.

## 4.2 Pilot Study

Before going to start thermochemical pretreatment and SSF with the current study, a pilot study was performed in which one year old stored wheat straw samples were used in order to optimize experimental conditions for optimum ethanol yield like design of bioreactor, TCP time, nitrogen flushing and SSF period. It was observed that 250ml bioreactor was not suitable for this study due to large head space and handling problems. So, a new 100ml bioreactor was designed for maximum ethanol yield with small head space and easy to handle. Ethanol concentration increased until 72 hours and then decreased most probably due to ethanol consumption of the yeast. It was due to presence of extra oxygen available due to large head space in 250 ml bioreactor. Moreover, 50 ml reaction mixture in 250 ml bioreactor was reduced to 35 ml for 100 ml bioreactor to avoid ethanol consumption as a carbon source due to presence of more oxygen in large head space.

## 4.3 Storage (ISP)

Fungal species (*Pichia anomala* and *Pichia stipitis*) were selected for ISP due to the fact that they were known to be good candidates for such type of studies (10). After one month storage with these species, some mould growth was observed in the falcons in which un-inoculated wet samples were stored at 15°C while there was no growth observed in the samples in which the yeast species were inoculated confirming their bio-preservative activity. No such growth was observed in dry wheat straw samples (figure 2, centre). *Pichia anomala* and *Pichia stipitis* (isolated from natural microorganism growing in un-inoculated sample) were growing on wheat straw agar plate (figure 2, right).

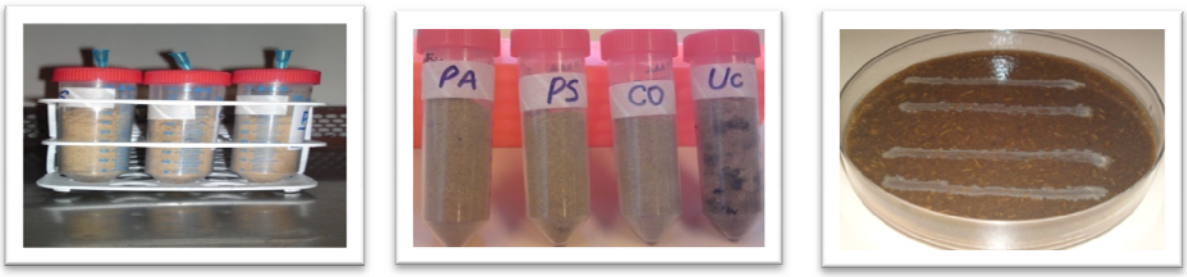
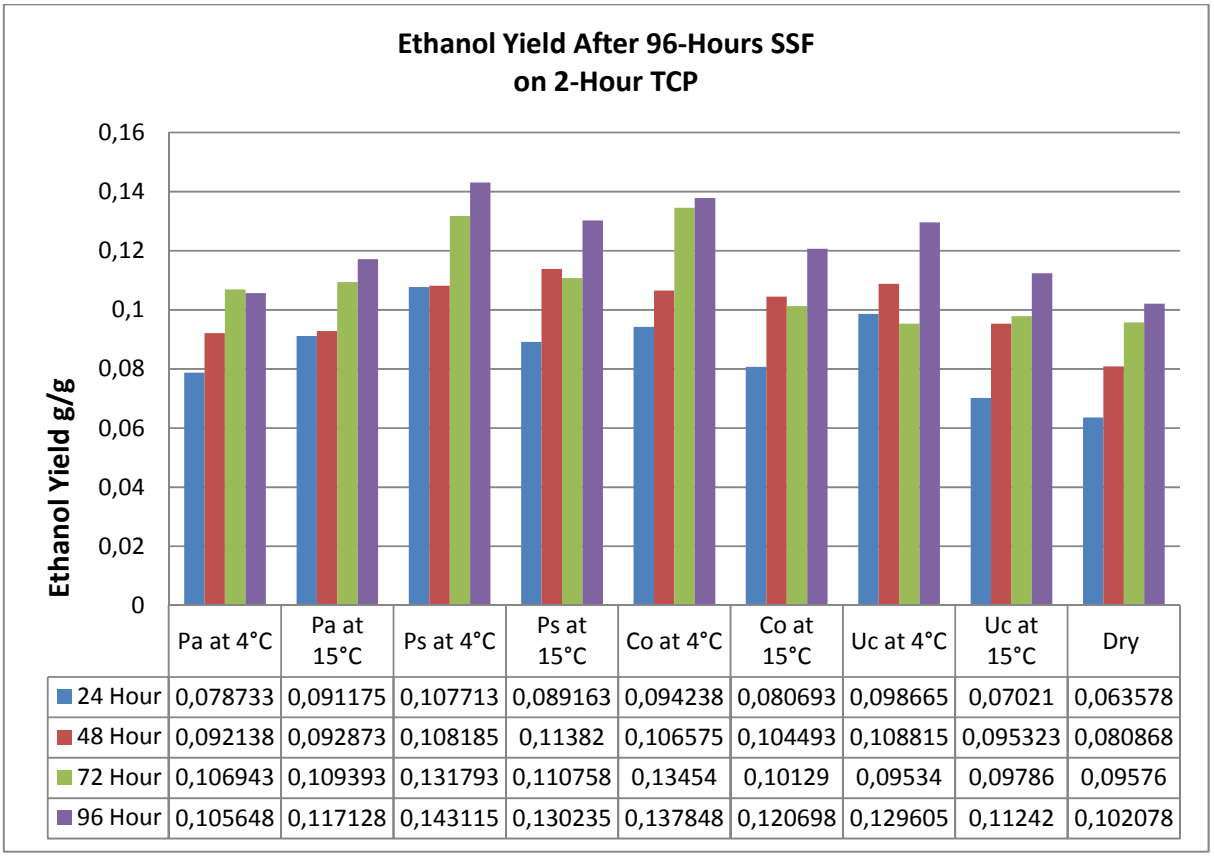



**Figure 3: Results after storage:** Yeast inoculated wheat straw samples during storage (left), after storage (centre) and yeast growth on wheat straw (right).

### 4.4 Ethanol Yield

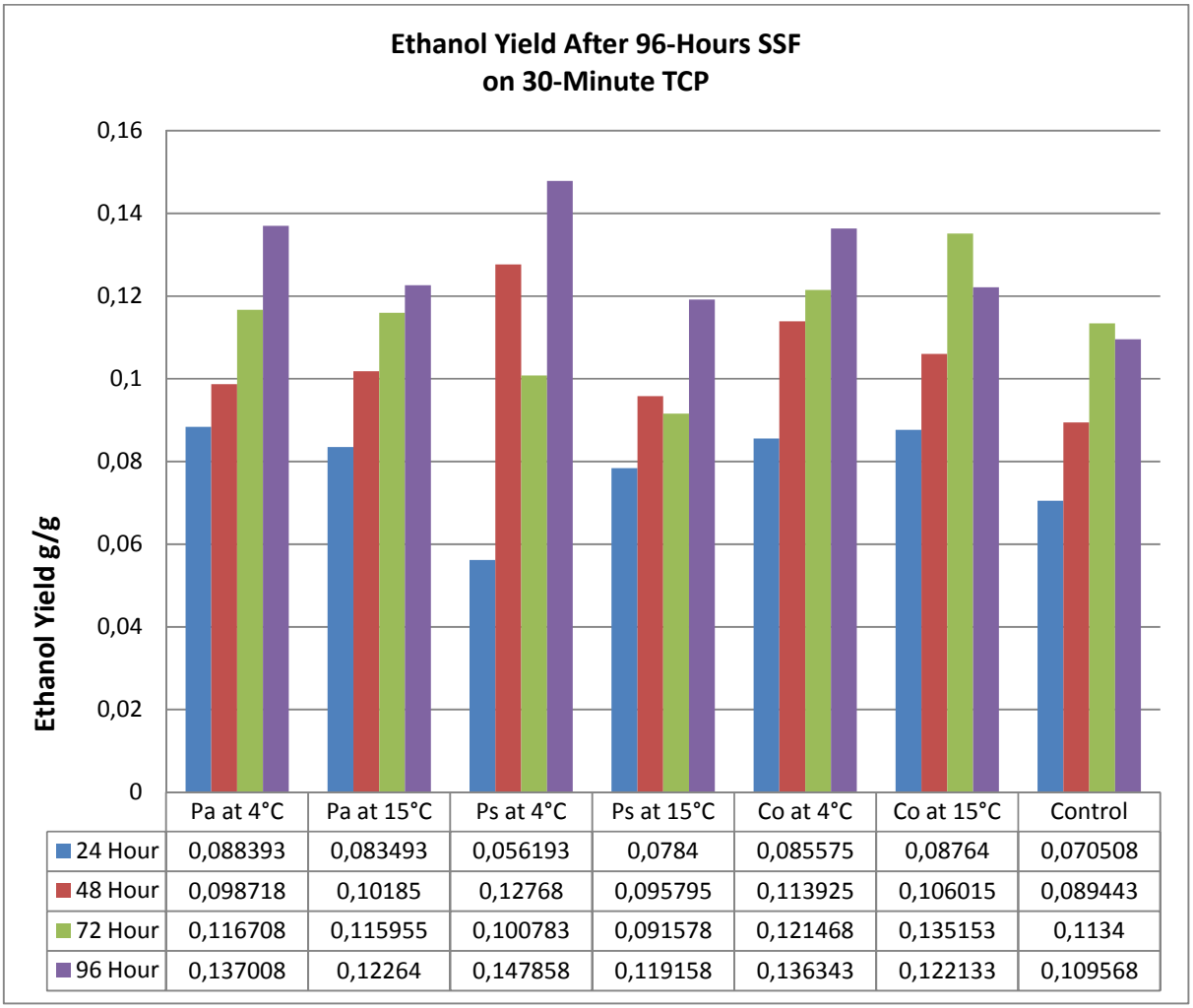
Yeast inoculated wheat straw samples and un-inoculated ones, along with dry wheat straw as a control, were analyzed after five days SSF. Samples were given 30 minute and 120 minute TCP before SSF in order to compare the effect of TCP time on ethanol yield. Ethanol yield was expressed in gram of ethanol per gram of dry weight as shown in figure 4 and 5.




| | Pa at 4°C | Pa at 15°C | Ps at 4°C | Ps at 15°C | Co at 4°C | Co at 15°C | Uc at 4°C | Uc at 15°C | Dry |
|---|---|---|---|---|---|---|---|---|---|
| 24 Hour | 0,078733 | 0,091175 | 0,107713 | 0,089163 | 0,094238 | 0,080693 | 0,098665 | 0,07021 | 0,063578 |
| 48 Hour | 0,092138 | 0,092873 | 0,108185 | 0,11382 | 0,106575 | 0,104493 | 0,108815 | 0,095323 | 0,080868 |
| 72 Hour | 0,106943 | 0,109393 | 0,131793 | 0,110758 | 0,13454 | 0,10129 | 0,09534 | 0,09786 | 0,09576 |
| 96 Hour | 0,105648 | 0,117128 | 0,143115 | 0,130235 | 0,137848 | 0,120698 | 0,129605 | 0,11242 | 0,102078 |

**Figure 4: Ethanol yield (g/g, after the storage) after 96-hour SSF on 2-Hours Thermo-chemical Pretreatment (TCP):** (P.a, P.s, Co, Uc and dry represent cultures inoculated with *Pichia anomala*, *Pichia stipitis*, co-culture of both species during storage, Un-inoculated control and dry (control) wheat straw samples, respectively) at high (15°C) and low temperature (4°C).

It was observed that at 2-hour TCP and after 4-day SSF, *Pichia stipitis* at 4°C gave 0.143g/g of ethanol that is 40.31% more as compared to control. Wheat straw stored with *Pichia stipitis* at higher temperature and of with co-culture of both species at low temperature also give better ethanol yield as compared to control. It was interesting finding that lower temperature was most suitable to soften the lignocellulosic structure than higher temperature. It may be due to limited growth of other natural microbial flora and better adaptation of this yeast species to low temperature.



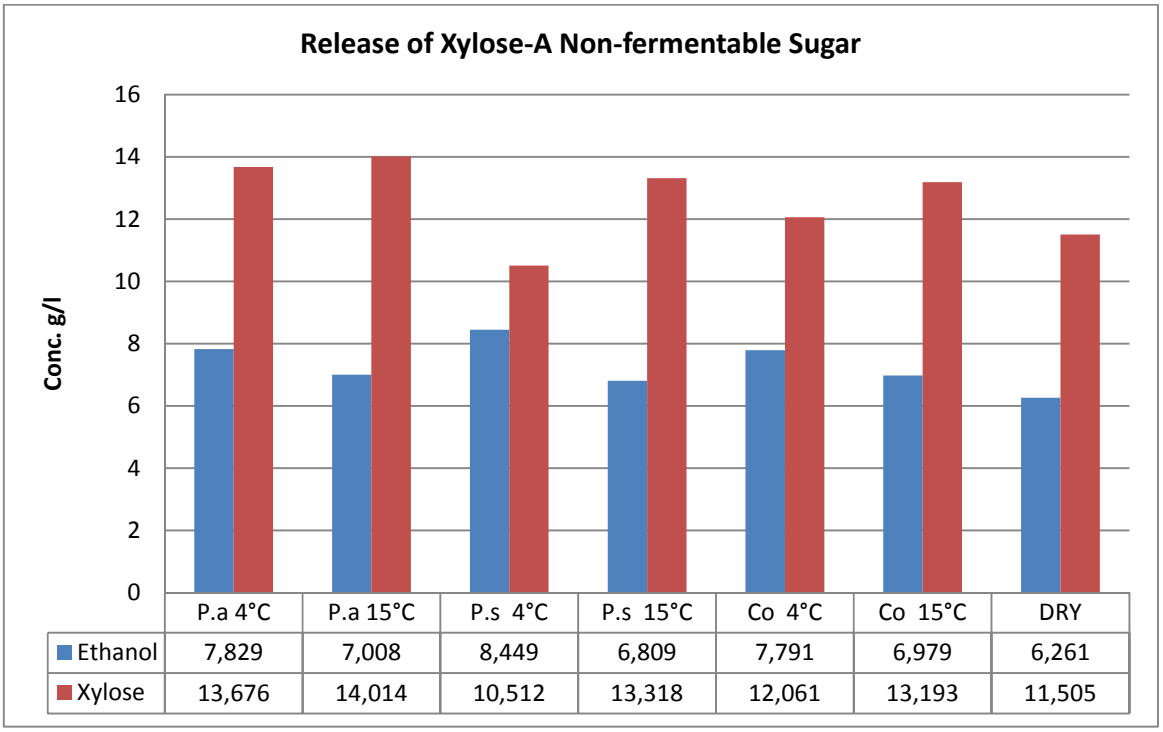

| | Pa at 4°C | Pa at 15°C | Ps at 4°C | Ps at 15°C | Co at 4°C | Co at 15°C | Control |
|---|---|---|---|---|---|---|---|
| 24 Hour | 0,088393 | 0,083493 | 0,056193 | 0,0784 | 0,085575 | 0,08764 | 0,070508 |
| 48 Hour | 0,098718 | 0,10185 | 0,12768 | 0,095795 | 0,113925 | 0,106015 | 0,089443 |
| 72 Hour | 0,116708 | 0,115955 | 0,100783 | 0,091578 | 0,121468 | 0,135153 | 0,1134 |
| 96 Hour | 0,137008 | 0,12264 | 0,147858 | 0,119158 | 0,136343 | 0,122133 | 0,109568 |

**Figure 5: Ethanol yield (g/g, after storage) after 96-hour SSF on 30-minute TCP:** P.a, P.s, Co, Uc and dry represent cultures inoculated with *Pichia anomala*, *Pichia stipitis*, co-culture of both species during storage, Un-inoculated control and dry (control) wheat straw samples, respectively at high (15°C) and low temperature (4°C).

In case of 30 minute of TCP, highest yield (0.147g/g) was measured again in the *Pichia stipitis* inoculated samples at 4°C confirming that low temperature was suitable for ISP. But, in contrary to 2-hour TCP (where *Pichia anomala* inoculated wheat straw sample produced lower amount of ethanol), *Pichia anomala* inoculated sample produced a bit higher ethanol at low temperature after 30 minute TCP.

### 4.5 Release of Non-fermented Sugars

Plant material was hydrolyzed by cellulase degrading enzymes that released pentose and hexose sugars during SSF. After glucose, xylose is the second abundant sugar released from biomass (14) and hence very important for biofuels production but *Saccharomyces cerevisiae* used in SSF in this study was unable to metabolize pentose sugars that were evident from the analysis (figure 6).



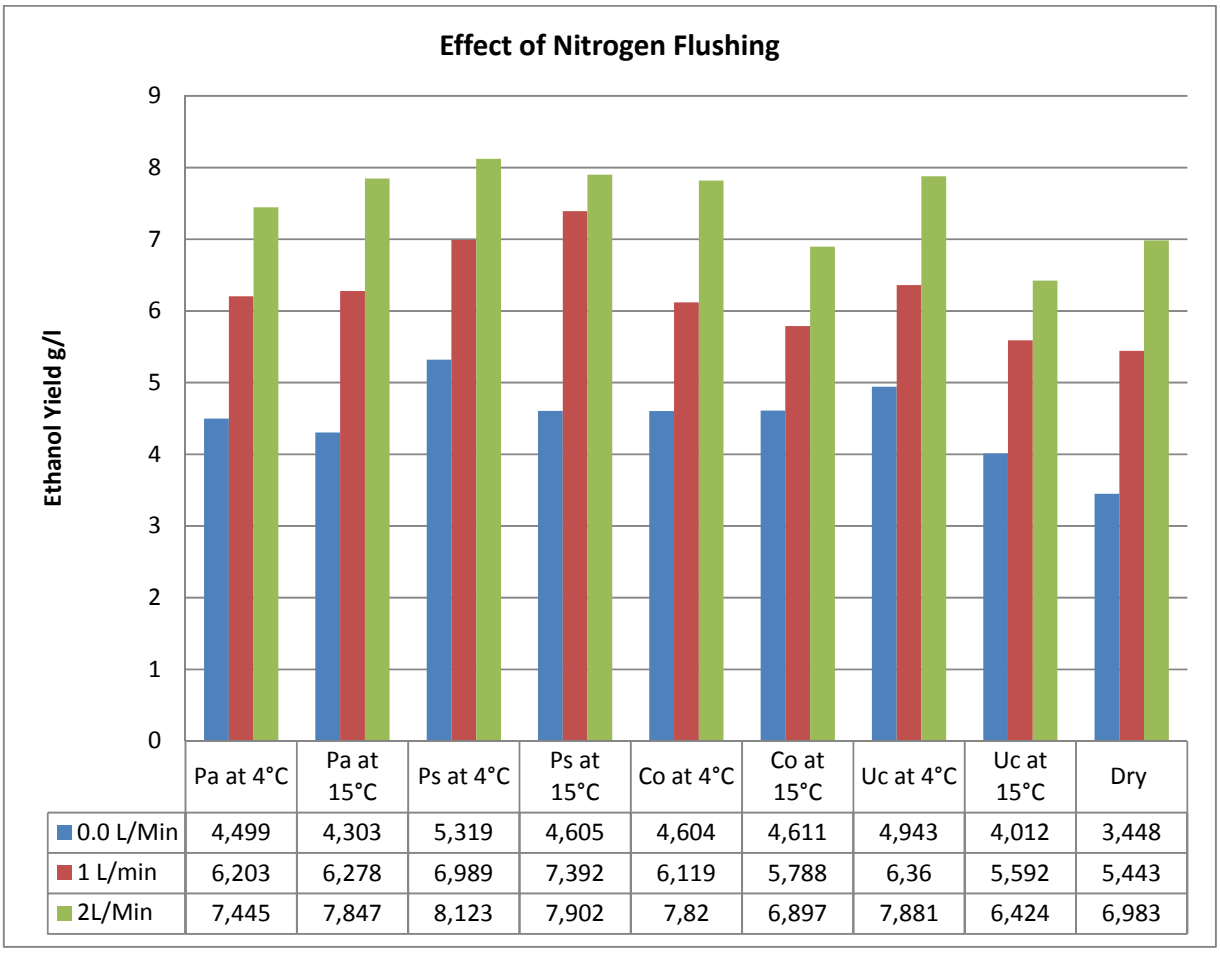

| | P.a 4°C | P.a 15°C | P.s 4°C | P.s 15°C | Co 4°C | Co 15°C | DRY |
|---|---|---|---|---|---|---|---|
| Ethanol | 7,829 | 7,008 | 8,449 | 6,809 | 7,791 | 6,979 | 6,261 |
| Xylose | 13,676 | 14,014 | 10,512 | 13,318 | 12,061 | 13,193 | 11,505 |

**Figure 6: Xylose and ethanol concentrations (g/l) after 96-hour SSF on 30-minutes TCP:** P.a, P.s, Co and dry represent cultures conserved with *Pichia anomala, Pichia stipitis,* co-culture of both species during storage and control wheat straw samples, respectively at low and high temperature.

It was clear from the above analysis that a reasonable amount of xylose was present in the hydrolysate which has further confirmed the efficiency of ISP because it has also indicated more de-crystallization of lignocellulosic biomass. More detailed analysis indicated that highest xylose concentration (14.01g/l) was released by *Pichia anomala* inoculated samples at higher temperature as compared to control. When xylose release was compared with ethanol yield, it was interesting to point out that *Pichia stipitis* inoculated samples produced highest ethanol (8.449g/l) but lowest amount of xylose (10.512g/l) as compared to others. Possibly, it is due to the fact that *Pichia stipitis* is an efficient xylose fermenting yeast and xylose released from hemicellulose could have been metabolized by the yeast during ISP. Moreover, glucose was not detected throughout the SSF confirming that glucose is efficiently taken up by the *Saccharomyces cerevisiae* and converted into ethanol.

### 4.6 Effect of Nitrogen Flushing

Importance of Nitrogen flushing was realized during the pilot study when 250 ml bioreactor was designed for optimization of TCP and SSF. It was observed that bioreactors that were flushed with more nitrogen produced more ethanol as compared to those which were not flushed but its confirmation required more experiments. Then, another 100ml bioreactor was designed in order to decrease head space because the ratio of head space was less (2/3) as compared to 250 ml bioreactors (4/5).




| | Pa at 4°C | Pa at 15°C | Ps at 4°C | Ps at 15°C | Co at 4°C | Co at 15°C | Uc at 4°C | Uc at 15°C | Dry |
|---|---|---|---|---|---|---|---|---|---|
| 0.0 L/Min | 4,499 | 4,303 | 5,319 | 4,605 | 4,604 | 4,611 | 4,943 | 4,012 | 3,448 |
| 1 L/min | 6,203 | 6,278 | 6,989 | 7,392 | 6,119 | 5,788 | 6,36 | 5,592 | 5,443 |
| 2L/Min | 7,445 | 7,847 | 8,123 | 7,902 | 7,82 | 6,897 | 7,881 | 6,424 | 6,983 |

**Figure 7: Effect of Ni trogen flushing on ethanol production (g/l) after 5-day SSF:** (P.a, P.s, Co and dry represent cultures conserved with *Pichia anomala*, *Pichia stipitis*, co-culture of both species and dry wheat straw samples during storage, respectively) at high (15°C) and low temperature (4°C).

It was an interesting finding that enhanced nitrogen flushing produced more ethanol and proved positive results as it was realized during pilot study for bioreactor designing and SSF optimization. So, keeping in view its effect on yield, in the current study samples were flushed with different doses of nitrogen (from 0.0l/minute to 2L/minute) as shown in the figure (7).

In the previous pilot study, SSF was almost completed and optimum yield was obtained within 96-hours but amount of ethanol was decreased after 72 hours due to consumption of ethanol as

on depletion of glucose. In the current study, nitrogen flushing was performed before SSF and an extra nitrogen treatment was given after 48 hours during SSF.

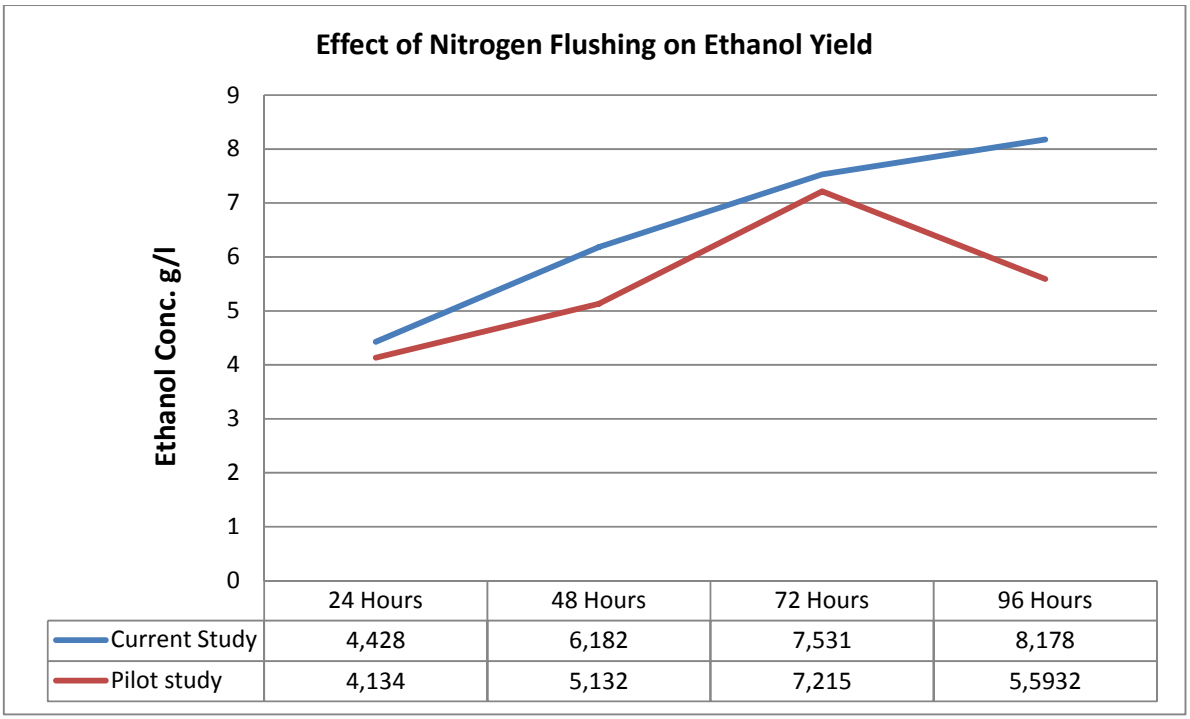



**Figure 8: Effect of Nitrogen flushing on ethanol production (g/l) after 5-day SSF:** *Pichia stipitis* inoculated wheat straw samples at low temperature (4°C) after 96 hour SSF. 1, 2, 3, 4 and 5 is the time scale in days.

In the end, it was observed that contrary to the pilot studies, ethanol production was increased after 72 hours and it was difficult to say when exactly ethanol formation would be completed. An increase in ethanol production after 72 hours may be due to less head space and complete anaerobic fermentation.

### 4.7 Effect of Thermo-chemical Pretreatment Time

It was hypothesized that ISP would decrease the TCP and increase the yield due to the partial de-crystallization of lignocellulosic biomass during ISP. It was observed that after inoculation with *Pichia stipitis* at low temperature for one month, TCP for 120 minutes and subsequent SSF at 35°C for 96 hours, 40.31% more ethanol was produced as compared to control dry wheat straw. On the other hand, 35.65% ethanol was produced as compared to control when treated with just 30 minutes while the other conditions were same and inoculation with the same yeast.

## CONCLUSION AND FUTURE PERSPECTIVE

It was demonstrated that integrated storage and pretreatment (ISP) is an efficient method for the production of fuel ethanol from lignocellulosic biomass.

It was observed that after inoculation of wheat straw samples with *Pichia stipitis* at low temperature (4°C) for one month storage, TCP for 120 minutes and subsequent SSF at 35°C for 96 hours, 40.31% more ethanol was produced as compared to control dry wheat straw. On the other hand, 35.65% ethanol was produced when treated with just 30 minutes while the other conditions were same and inoculation with the same yeast.

By using other lignocellulosic biomass, optimizing TCP time and conditions, technically and optimally well designed automated bioreactors, using engineered yeast that would be able to ferment mixed sugars and exploring more potent microorganisms from natural environment, ISP would be a breakthrough for the production of biofuels from lignocellulosic biomass.

## ACKNOWLDGEMENT

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I pay special thanks to my family members who are praying for me specially my beloved wife, who is thousand miles away but rests in my heart and soul.

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# APPENDIX

**Table 1: Results of SSF a fter ISP at 4°C after 30 minutes of thermo-chemical pretreatment. (P.a, P.s, Co, and control represent wheat straw samples inoculated with *Pichia anomala, Pichia stipitis,* co-culture of both species during storage and dry (control) wheat straw samples, respectively)**

| SSF | Sample | Xylose (g/l) | Ethanol (g/l) | Ethanol (g/g) |
|---|---|---|---|---|
| After 24 hours SSF | P.a | 13.676 | 5.051 | 0.088393 |
| | P.s | 10.512 | 3.211 | 0.056193 |
| | Co | 12.061 | 4.89 | 0.085575 |
| | Control | 11.505 | 4.029 | 0.070508 |
| After 48 hours SSF | P.a | 14.757 | 5.641 | 0.098718 |
| | P.s | 15.998 | 7.296 | 0.12768 |
| | Co | 13.696 | 6.51 | 0.113925 |
| | Control | 12.005 | 5.111 | 0.089443 |
| After 72 hours SSF | P.a | 14.565 | 7.829 | 0.137008 |
| | P.s | 15.716 | 8.449 | 0.147858 |
| | Co | 13.468 | 7.791 | 0.136343 |
| | Control | 11.685 | 6.261 | 0.109568 |
| After 96 hours SSF | P.a | 13.326 | 6.669 | 0.116708 |
| | P.s | 10.983 | 5.759 | 0.100783 |
| | Co | 13.099 | 6.941 | 0.121468 |
| | Control | 14.01 | 6.48 | 0.1134 |

**Table 2: Results of SSF after ISP at 15°C after 30 minutes of thermo-chemical pretreatment. (P.a, P.s, Co, and control represent wheat straw samples inoculated with *Pichia anomala, Pichia stipitis,* co-culture of both species and dry wheat straw samples, respectively)**

| SSF | Sample | Xylose (g/l) | Ethanol (g/l) | Ethanol (g/g) |
|---|---|---|---|---|
| After 24 hours SSF | P.a | 14.014 | 4.771 | 0.083493 |
| | P.s | 13.318 | 4.48 | 0.0784 |
| | Co | 13.193 | 5.008 | 0.08764 |
| | Control | 11.505 | 4.029 | 0.070508 |
| After 48 hours SSF | P.a | 13.35 | 5.82 | 0.10185 |
| | P.s | 13.103 | 5.474 | 0.095795 |
| | Co | 13.143 | 6.058 | 0.106015 |
| | Control | 12.005 | 5.111 | 0.089443 |
| After 72 hours SSF | P.a | 13.761 | 7.008 | 0.12264 |
| | P.s | 13.466 | 6.809 | 0.119158 |
| | Co | 13.075 | 6.979 | 0.122133 |
| | Control | 11.685 | 6.261 | 0.109568 |
| After 96 hours SSF | P.a | 14.643 | 6.626 | 0.115955 |
| | P.s | 10.664 | 5.233 | 0.091578 |
| | Co | 13.871 | 7.723 | 0.135153 |
| | Control | 14.01 | 6.48 | 0.1134 |

**Table 3: Results of SSF after integrated storage of wheat straw (ISP) after 120 minutes of thermo-chemical pretreatment at 4°C. (P.a, P.s, Co, Uc and dry represent cultures inoculated with *Pichia anomala, Pichia stipitis,* co-culture of both species during storage, Un-inoculated control and dry (control) wheat straw samples, respectively)**

| SSF | Sample | Xylose (g/l) | Ethanol (g/l) | Ethanol (g/g) |
|---|---|---|---|---|
| After 24 hours SSF | P.a | 12.132 | 4.499 | 0.078733 |
| | P.s | 15.92475 | 6.155 | 0.107713 |
| | Co | 13.8895 | 5.385 | 0.094238 |
| | Un | 14.4305 | 5.638 | 0.098665 |
| | Dry | 12.058 | 3.633 | 0.063578 |
| After 48 hours SSF | P.a | 12.042 | 5.265 | 0.092138 |
| | P.s | 13.128 | 6.182 | 0.108185 |
| | Co | 13.358 | 6.09 | 0.106575 |
| | Un | 12.827 | 6.218 | 0.108815 |
| | Dry | 12.6275 | 4.621 | 0.080868 |
| After 72 hours SSF | P.a | 12.612 | 6.111 | 0.106943 |
| | P.s | 14.36 | 7.531 | 0.131793 |
| | Co | 14.4235 | 7.688 | 0.13454 |
| | Un | 9.886 | 5.448 | 0.09534 |
| | Dry | 12.8605 | 5.472 | 0.09576 |
| After 96 hours SSF | P.a | 11.6615 | 6.037 | 0.105648 |
| | P.s | 14.605 | 8.178 | 0.143115 |
| | Co | 13.669 | 7.877 | 0.137848 |
| | Un | 13.21 | 7.406 | 0.129605 |
| | Dry | 12.2895 | 5.833 | 0.102078 |
| | | | | |

**Table 4: Results of SSF after integrated storage of wheat straw (ISP) after 120 minutes of thermo-chemical pretreatment at 15°C. (P.a, P.s, Co, Uc and dry represent cultures inoculated with *Pichia anomala, Pichia stipitis,* co-culture of both species du ring storage, Un-inoculated control and dry (control) wheat straw samples, respectively)**

| SSF | Sample | Xylose (g/l) | Ethanol (g/l) | Ethanol (g/g) |
|---|---|---|---|---|
| After 24 hours SSF | P.a | 14.3715 | 5.21 | 0.091175 |
| | P.s | 13.366 | 5.095 | 0.089163 |
| | Co | 13.8975 | 4.611 | 0.080693 |
| | Un | 11.918 | 4.012 | 0.07021 |
| | Control | 12.058 | 3.633 | 0.063578 |
| After 48 hours SSF | P.a | 12.12 | 5.307 | 0.092873 |
| | P.s | 13.996 | 6.504 | 0.11382 |
| | Co | 13.9855 | 5.971 | 0.104493 |
| | Un | 13.293 | 5.447 | 0.095323 |
| | Control | 12.6275 | 4.621 | 0.080868 |
| After 72 hours SSF | P.a | 12.1795 | 6.251 | 0.109393 |
| | P.s | 12.1435 | 6.329 | 0.110758 |
| | Co | 11.1865 | 5.788 | 0.10129 |
| | Un | 11.819 | 5.592 | 0.09786 |
| | Control | 12.8605 | 5.472 | 0.09576 |
| After 96 hours SSF | P.a | 11.785 | 6.693 | 0.117128 |
| | P.s | 13.6175 | 7.442 | 0.130235 |
| | Co | 13.335 | 6.897 | 0.120698 |
| | Un | 11.917 | 6.424 | 0.11242 |
| | Control | 12.2895 | 5.833 | 0.102078 |
| | | | | |