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Adjustment of Yeast Growth Media for the Fermentation of Lignocellulosic Sugars

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Efficient ethanol production from lignocellulosic biomass is closely linked to the selected pre-treatment/hydrolysis technique, fermentable sugar yield and composition, microorganism of choice and presence of inhibitory compounds. In this study the applicability of lignocellulosic hydrolysates was evaluated with respect to the overall cell yields and sugar consumption rate. The effect of most popular additional nutrients, like, yeast extract and peptone, was assessed. Fermentable sugar hydrolysates were prepared with simple mechanical pre-treatment and enzymatic hydrolysis. The results showed that nutrient media or biomass hydrolysate gave similar *Saccharomyces cerevisiae* biomass increase, 2.7 and 2.34 log increase after 48 hours respectively. At the same time sugar consumption rate in nutrient media was more than 3 times faster than in hydrolysates. Addition of extra C6 carbohydrate to the hydrolysate increased sugar consumption rate and cell biomass yields to only a certain extent, afterwards it became inhibitory. Concentration of reducing sugars, did not resulted into higher efficiency of hydrolysates, however, the results showed that peptone and yeast extract did not have any significant ($p > 0.05$) effect on cell growth or sugar consumption. Moreover, hydrolysate showed to be a good base medium when opposed to clean water. In conclusion, the results demonstrated that pure enzymatically hydrolysed biomass can be used as a single resource for yeast fermentation.

1. Introduction

1st generation bio-ethanol is produced from energy crops via fermentation of hydrolysed starch or sugar from sugarcane. Debates on the use of agricultural land for energy crops have set the need to evaluate and apply other materials, e.g., lignocellulosic biomass. Up to date, the suitability of a wide range of materials, e.g. forest and agriculture residues, has been tested for ethanol production (Limayem and Ricke, 2012; Sanchez and Cardona, 2008). Similarly, tremendous amounts of technologies for pre-treatment and hydrolysis of the biomass have been suggested over the years (Kumar et al., 2009). At the same time, to decrease ethanol production costs, research on technological design of the fermentation process and potential inhibitors is still ongoing (Jönsson et al., 2013; Liu et al., 2016; Sanchez and Cardona, 2008).

Excellent biomass hydrolysate should contain no inhibitory substances and high enough concentration of fermentable sugars. Combination of enzymatic hydrolysis with an appropriate pre-treatment and or detoxification technology has enabled to decrease the amount of fermentation inhibitors (Jönsson and Martin, 2016), application of specific or genetically modified ethanol producing microorganisms has solved issues in *Saccharomyces* inability to ferment C5 sugars (Sanchez and Cardona, 2008) or introduction of simultaneous saccharification and fermentation has been proposed as a complex approach (Kádár et al., 2004). Still, sugar yields are strongly affected by the applied enzymes and hydrolysis conditions (Yang et al., 2011). Mild pre-treatment is seen as a limitation for enzyme accessibility (Kumar et al., 2009; Yang et al., 2011), however, this can be effectively solved with innovative engineering approaches (Dalecka et al., 2015) or further processing of waste generated during the hydrolysis.

Nevertheless, efficient alcohol production is not limited to carbon source only. To obtain optimal yields, other essential elements (nitrogen, phosphorous and vitamins) need to be supplied with the hydrolysate as chemicals added during or before hydrolysis (Chang and Webb, 2017) or they need to be added to the fermentation media. Often laboratory scale research on ethanol fermentation is achieved by supplying the

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media with yeast extract or peptones at relatively high concentrations (Chen et al., 2007; Dussan et al., 2014). At the same time, this contradicts to the need for the development of a cheap and efficient technology. The aim of this research was to determine if nutrient supplements are necessary for *Saccharomyces* growth and sugar consumption in lignocellulosic hydrolysates that are produced with simple pre-treatment and enzymatic hydrolysis technology. To obtain hydrolysates with higher sugar concentration pilot scale membrane technologies were introduced into preparation of the hydrolysates.

2. Materials and Methods

2.1 Microorganism and inoculum preparation

Saccharomyces cerevisiae (Compressed Baker`s yeast, Lithuania) grown on YPD agar (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L, agar 15 g/L) was subcultured into liquid (agar-free) YPD and grown for 24 hours at 30°C. Afterwards the cells were twice washed (centrifugation for 2 min at 2,000 g, Eppendorf MiniSpin Plus) with sterile phosphate buffered saline (PBS) and re-suspended in PBS to get around 10^6 cells per mL.

Yeast cell concentration was determined by staining with DAPI (4',6-diamidino-2-phenylindole, Merck). In brief, a known volume of sample (adjusted to obtain15–100 cells per microscope field of view) was filtered onto 25 mm diameter, 0.2 μm pore-size filters (Nucleopore Track-Etched; Whatman plc), fixed with 3–4% (*v*/*v*) formaldehyde and stained with10 μg/mL DAPI for 15 min. Cell numbers were determined by epifluorescencemicroscopy by counting 20 random fields of view (Ex.340/380 nm; Em. >425 nm, dichromatic mirror 565 nm, Leica 6000B). Cell concentration was expressed as cells/mL.

2.2 Preparation of hydrolysates

Lignocellulosic biomass hydrolysates (fermentable sugars) were prepared from hay biomass (collected in Latvia, 2015, DW 92.8 ± 1.3%) that was grinded to fractions < 0.5 cm. 3% w/v of the biomass was diluted in 0.05 M sodium citrate buffer (mono–sodium citrate pure, AppliChem, Germany) and boiled for 5 min (1 atm) to eliminate any indigenous microorganisms. After cooling to room temperature an enzyme (Viscozyme, Novozymes) was added to the diluted samples and incubated on an orbital shaker (New Brunswick, Innova 43) for 24 h at 37 °C and 150 rpm. Sugar concentration in hydrolysates varied from 4.8 – 8.4 g/L. To obtain hydrolysates with higher sugar concentrations (above 20 g/L) hydrolysis was performed at a pilot scale system equipped with membranes (Dalecka et al., 2015).

2.3 Growth studies

Growth studies were performed in 100 mL laboratory flasks filled with growth media (Hydrolysate, concentrated hydrolysate, hydrolysate with added dextrose, yeast extract or peptone at various concentrations) to which *S. cerevisiae* cell suspension was added to produce a final concentration of 10^3 – 10^4 cells/mL. Then the samples were incubated on an orbital shaker (150 rpm) at 30°C. Samples for yeast cell concentration (staining with DAPI) and sugar consumption analyses were collected in duplicates after 24 and 48 hours of incubation. All sets of experiments contained samples with YPD and sterile distilled water as positive and negative control respectively.

2.4. Reducing sugar analyses

Reducing sugar analysis for all collected samples was performed using Dinitrosalicylic Acid (DNS) method (Ghose, 1987). Before testing all samples were centrifuged (6600 g, 10 min). Then 0.1 mL of the supernatant was mixed with 0.1 mL of 0.05 M sodium citrate buffer and 0.6 mL of DNS (SigmaAldrich, Germany). For blank control, distilled water was used instead of the sample. Then all samples were boiled for 5 min and transferred to cold water. Next 4 mL of distilled water was added. Absorption was measured with spectrophotometer M501 (Camspec, United Kingdom) at 540 nm. To obtain absolute concentrations, a standard curve against glucose was constructed. Sugar consumption rate was expressed as $LOG(C/C_0)$.

2.5. Statistical analyses

MS Excel 2007 t–test (two tailed distribution) and ANOVA single parameter tool (significance level ≤ 0.05) were used for analysis of variance on data from various sample setup`s.

3. Results and Discussions

Efficient ethanol fermentation from lignocellulosic hydrolysates is traditionally linked to sugar concentration, amount of C6 sugars, concentration of inhibitory substances and production costs (Sanchez and Cardona, 2008). Thus, laboratory praxis of supplementing the fermentation broth with expensive, nutrient rich chemicals

cannot be introduced into full scale ethanol production. In this study the necessity and impact of peptone, yeast extract and additional carbon source was evaluated along to the overall influence of lignocellulosic hydrolysates obtained via enzymatic hydrolysis. Peptone and yeast extract was used as sources of amino acids, nitrogen, phosphorous and vitamins essential for growth of all living cells. To minimise the impact of inhibitors, hydrolysates were produced with a mild pre-treatment (only mechanical size reduction and boiling to remove indigenous microorganisms) (Mezule et al., 2015) and enzymatic hydrolysis. The obtained reducing/fermentable sugar yields ranged from 6 – 8.4 g/L. Cell growth assessment showed that there is only a slight ($p > 0.05$) difference in-between cell biomass yields from hydrolysates and nutrient media (YPD) - 2.34 and 2.7 log after 48 hours respectively (Figure 1). At the same time sugar consumption was much lower in samples containing only hydrolysate (Table 1) and all samples still contained some amount of reducing sugar (0.6 – 2 mg/L) after 48 h of incubation that could be explained as the un-fermentable C5 carbohydrates (Chang and Webb, 2017), thus, confirming the potential bottlenecks of the application of classic *S. cerevisiae* in lignocellulose conversion (Limayem and Ricke, 2012).

Addition of low concentration of peptone supplement showed no impact on cell growth ($p > 0.05$) or sugar consumption rate, which after 48 h ranged from -0.60 to -0.69. Moderate supplement of carbon source (50 g/L dextrose) resulted in an increased cell growth (2.65 log after 48 h) but no increase in sugar consumption rate was observed. At the same time, high cell yields (2.42 log after 48 h) and rapid sugar consumption (-1.41) was achieved when the hydrolysates were supplemented with both 50 g/L dextrose and 0.1 g/L peptone. Further, an increase in dextrose concentration (100 g/L) did not produce any significant effect. Moreover, a sharp decrease in sugar consumption rate was observed.

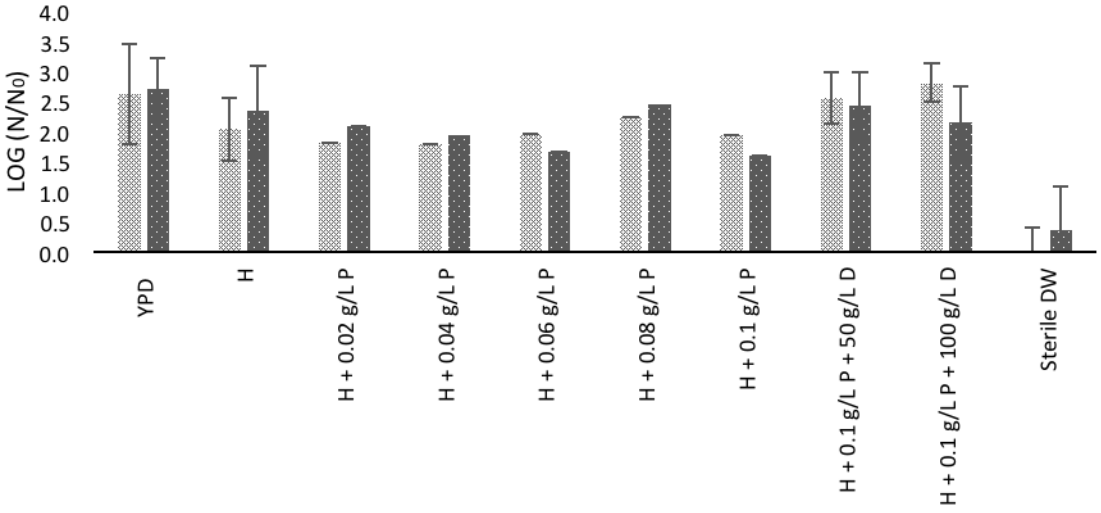

Figure 1: The effect of peptone (P) and additional carbon source (D, as dextrose) on S. cerevisiae cell growth in hydrolysates after 24 (light) and 48 (dark) of incubation. Yeast-Peptone-Dextrose (YPD) broth and sterile distilled water (DW) were used as positive and negative controls. Each bar represents an average value of at least three separate measurements.

A potential inhibition of the sugar consumption rate by the hydrolysates was observed. Faster sugar consumption was observed in slightly diluted hydrolysates (Table 1). A decrease was seen when water in YPD was substituted with a hydrolysate (from -1.79 to -0.55). At the same time no difference ($p > 0.05$) in cell growth was observed in any of the samples and ranged from 2.14 (90 % hydrolysate) to 2.7 (YPD) log increase after 48 h of incubation indicating on potential presence of chemicals that affect cell growth but do not affect respiratory / fermentative metabolism.

The evaluation of the hydrolysate as a potential source of other essential chemicals showed that there is a strong decrease in cell growth in samples when the hydrolysate is substituted with distilled water (DW). A very low cell increase (0.49 log after 48 h) was seen in samples which contained 50 g/L dextrose in sterile DW. At the same time 50 g/L dextrose in the hydrolysate yielded 2.65 log cell increase. Furthermore, lower cell yields were obtained in samples with 50 g/L dextrose and 0.1 g/L peptone in DW (1.91 log after 48 h) than in hydrolysate (2.42 log after 48 hours). The same trend was observed in sugar consumption analyses where practically no consumption of dextrose was observed in samples containing DW instead of the hydrolysate (Table 1). Thereby it confirms to the previous observations that essential nutrients can come from the

lignocellulosic biomass (Kurian et al., 2010) or from the enzyme leftovers. A minor increase in sugar consumption and overall biomass yields was observed for samples incubated in pure DW. One of explanation could be the consumption of nutrients from dead cells.

Table 1: Sugar consumption rates in various samples.

Sample type	Sugar consumption rate(LOG(C/C_o))	
	24 h	48 h
YPD	-2.09 ± 0.31	-1.79 ± 0.26
Hydrolysate	-0.59 ± 0.13	-0.57 ± 0.04
90 % Hydrolysate	-0.71 ± 0.23	-0.84 ± 0.41
concentrated Hydrolysate	-0.17 ± 0.15	-0.52 ± 0.05
YPD + Hydrolysate	-0.52 ± 0.14	-0.55 ± 0.14
Hydrolysate + 50 g/L dextrose	-0.03 ± 0.06	-0.32 ± 0.01
Sterile DW + 50 g/L dextrose	0.03 ±0.06	0 ± 0.08
Hydrolysate + 50 g/L dextrose + 0.1 g/L peptone	-0.16 ± 0.12	-1.41 ± 0.58
Hydrolysate + 100 g/L dextrose + 0.1 g/L peptone	0.02 ± 0.05	-0.17 ± 0.09
Sterile DW + 50 g/L dextrose + 0.1 g/L peptone	0 ± 0.06	-0.02 ± 0.1
Sterile DW + 100 g/L dextrose + 0.1 g/L peptone	0.02 ± 0.04	0.02 ± 0.04
concentrated Hydrolysate + 50 g/L dextrose	-0.03 ± 0.07	- 0.96 ± 0.01
concentrated Hydrolysate + 100 g/L dextrose	-0.11 ± 0.08	-0.79 ± 0.00
sterile DW + 50 g/L dextrose	0.03 ± 0.06	0 ± 0.08

To further evaluate the impact of the hydrolysate and sugar as fermentation limiting factor, hydrolysate concentration with a complex membrane system was performed (Dalecka et al., 2015) and resulted in reducing sugar concentration above 20 g/L. Cell growth studies demonstrated that there is an increase in cell biomass yields (Figure 2) and the impact of prolonged incubation period was confirmed even more than in un-concentrated hydrolysates. Sugar consumption rates after 48 hours were similar in both concentrated and regular hydrolysates (Table 1). Slower conversion of reducing sugars in hydrolysates could be linked with the decrease of oxygen in the fermentation media, thus, creating a more favourable environment for solvent production.

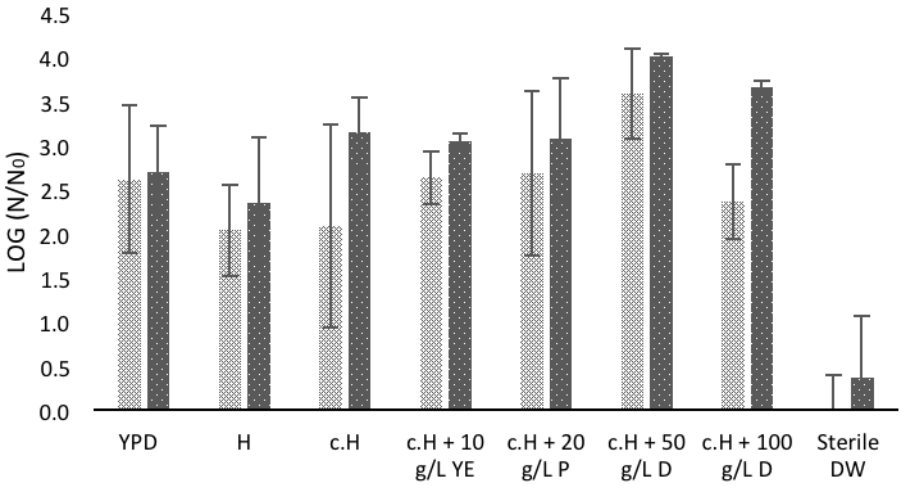

Figure 2: The effect of concentrated hydrolysate and additional supplements (YE as yeast extract; P as peptone and D as dextrose) on S. cerevisiae cell growth after 24 (light) and 48 (dark) hours of incubation. Yeast-Peptone-Dextrose (YPD) broth and sterile distilled water (DW) were used as positive and negative controls. Each bar represents an average value of at least three separate measurements.

Addition of peptone or yeast extract to concentrated hydrolysates did not generated higher biomass yields ($p > 0.05$) and no effect on overall sugar consumption rate was observed ($p > 0.05$) which ranged from -0.41 to -0.43 after 48 hours. At the same time the addition of 50 g/L dextrose increased sugar consumption rate (-0.96)

and produced higher amount of cell biomass. Further increase in C6 sugar concentration showed no significant increase in cell biomass and sugar consumption rates. Moreover, even some inhibition was observed as a result of lower biomass increase and low sugar consumption rate.
Microscopy examination showed that cells in concentrated hydrolysates were less bright (lower DNA content) and samples contained relatively high amount of cell debris (Figure 3D). At the same time no decrease in cell size or any other shape irregularity was observed. Addition of extra C6 sugar (Figure 3C) yielded higher cell counts and resulted in very bright to pale cells in a single sample indicating on apparent inability of all cells to survive in lignocellulosic hydrolysates.

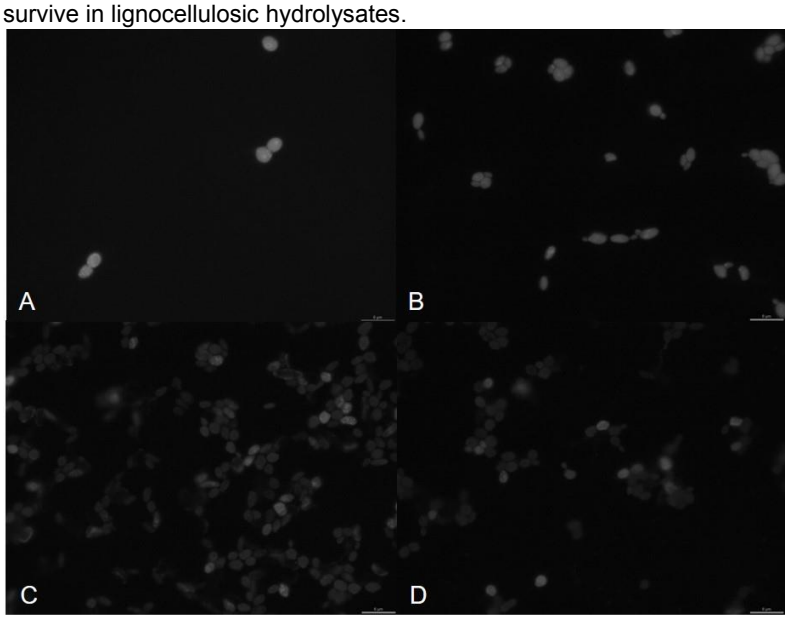

Figure 3: Initial inoculum (A) and changes in cell appearance after 48 hours of incubation in YPD media (B), concentrated hydrolysate (D) and concentrated hydrolysate supplemented with 50 g/L dextrose (C). Bar represents 6 µm. All samples except initial inoculum had 0.1 ml test volume. Initial inoculum – 1 ml.

Lignocellulosic biomass is regarded as a potential resource for alcohol production, however, its efficiency strongly depends on the pre-treatment/hydrolysis technology (Singh et al., 2014). Chemical treatment is well known to produce toxic inhibitors and require additional treatment prior fermentation. Enzymatic hydrolysis at the same is regarded as environmentally friendly technology but with slow conversion rates and high dependency of the applied enzymes (Limayem and Ricke, 2012). In this study, tests of enzymatically hydrolysed biomass showed that, to grow yeast, there is no need to add other nutrient sources, like peptone or yeast extract. The hydrolysate apparently contains enough nitrogen and phosphorous from the biomass or enzyme leftovers. At the same time not all available sugar was consumed by the yeast cells. The apparent presence of C5 sugars indicated on the need to use other microorganism or use the fermentation waste for further energy production, e.g., biogas.
Addition of C6 carbohydrate resulted in faster sugar consumption what is often linked with higher alcohol yields (Otterstedt et al., 2004) and lower cell biomass. Thus, a more sophisticated hydrolysate concentration technology and potential separation of C5 and C6 sugars could be a reasonable approach in future applications. Nevertheless, when total sugar concentration exceeded a certain threshold it became both inhibiting to cell growth and decreased the overall sugar consumption rates.

4. Conclusions

The results of the study showed that there is no significant impact of peptone or yeast extract on *S. cerevisiae* growth and sugar consumption rates in lignocellulosic hydrolysates. Moderate increase in sugar consumption rate and yeast biomass yield was observed after increasing the amount of C6 carbon source which pointed to the need to introduce a more sophisticated fermentable sugar separation technology or application of another fermentative microorganism. Consumption of sugar from hydrolysates was slower than for pure C6 carbohydrate, nevertheless, the data showed that lignocellulosic biomass can be used as a single growth medium in yeast ethanol production.

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References

Chen M., Xia L. and Xue P., 2007, Enzymatic hydrolysis of corncob and ethanol productionfrom cellulosic hydrolysate, Int. Biodeterior. Biodegradation, 59, 85-89.

Chang C.W. and Webb C., 2017, Production of a generic microbial feedstock for lignocellulose biorefineries through sequential bioprocessing, Bioresour Technol., 227, 35-43.

Dalecka B., Strods M. and Mezule L., 2015, Production of fermentation feedstock from lignocellulosic biomass: applications of membrane separation, Agron. Res., 13, 287-293.

Dussan K.J., Silva D.D.V., Moraes E.J.C., Arruda P.V. and Felipe M.G.A., 2014, Dilute-acid hydrolysis of cellulose to glucose from sugarcane bagasse, Chemical Engineering Transactions, 38, 433-438.

Ghose T.K., 1987, Measurement of cellulose activities, Pure&Appl. Chem., 59, 257-268.

Jönsson L.J., Alriksson B. and Nilvebrant N.O., 2013, Bioconversion of lignocellulose: inhibitors and detoxification, Biotech. Biofuels, 6:16, 1-10.

Jönsson L.J. and Martín C., 2016, Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects, Bioresour. Technol., 199, 103-112.

Kádár Z., Szengyel Z. and Réczey K., 2004, Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol, Ind. Crops Prod., 20,103-110.

Kumar P., Barrett D.M., Delwiche M.J. and Stroeve P., 2009, Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production, Ind. Eng. Chem. Res., 48, 3713-3729.

Kurian J.K., Minu A.K., Banerji A. and Kishore V.V.N., 2010, Bioconversion of hemicellulose hydrolysate of sweet sorghum bagasse to ethanol by using *Pichia stipites* NCIM 3497 and *Debaryomyces hansenii* sp., BioResources, 5, 2404-2416.

Limayem A. and Ricke S.C., 2012, Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects, Prog. Energy Comb. Sci., 38, 449-467.

Liu X., Xy W., Mao L., Zhang C., Yan P., Xu Z. and Zhang Z.C., 2016, Lignocellulosic ethanol production by starch-base industrial yeast under PEG detoxification, Sci. Rep., 6, 1-11.

Mezule L., Dalecka B. and Juhna T., 2015, Fermentable sugar production from lignocellulosic waste, Chemical Engineering Transactions, 43, 619-624.

Otterstedt K., Larsson C., Bill R. M., Ståhlberg A., Boles E., Hohmann S. and Gustafsson L., 2004, Switching the mode of metabolism in the yeast *Saccharomyces cerevisiae*, EMBO Rep., 5, 532–537.

Sanchez O.J. and Cardona C.A., 2008, Trends in biotechnological production of fuel ethanol from different feedstocks, Bioresour. Technol., 99, 5270-5295.

Singh R., Shukla A., Tiwari S. and Srivastava M., 2014, A review on delignification of lignocellulosic biomass for enhancement of ethanol production potential, Renew. Sust. Energ. Rev., 32, 713-728.

Yang B., Dai Z., Ding S.Y. and Wyman C.E., 2011, Enzymatic hydrolysis of cellulosic biomass, Biofuels, 2(4), 421-450.