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BIOETHANOL PRODUCTION FROM THERMOCHEMICALLY PRE-TREATED OLIVE MILL SOLID RESIDUES USING THE YEAST *Pachysolen tannophilus*

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ABSTRACT

Olive oil mill solid residue (OMSR) is the solid waste generated during olive oil production process in three-phase olive mills. It consists of the remaining pulp of olive processing after the extraction of oil, as well as the cracked seeds of the olive fruits, containing thus mainly lignocellulose and residual oil. The commonly used practice for OMSR management is combustion, after having extracted the residual oil by secondary extraction using organic solvents. Other proposed ways of OMSR management are their exploitation as substrate for edible fungi production and compost, and as feedstock for biofuels generation such as methane and bioethanol. In the latter case, the complex carbohydrates (cellulose and hemicellulose) of the lignocellulose of OMSR have to be degraded towards their simple sugars and further fermented via microorganisms.

The purpose of the present study was to investigate the effect of thermochemical pre-treatment of OMSR, on the final ethanol yield from the yeast *Pachysolen tannophilus*. Nine different types of OMSR-based substrates were tested i.e. raw OMSR, hydrolysates generated from pretreated OMSR with NaOH (0.5 %, 1.5 % w/v) and H_2SO_4 (0.5 %, 1.5 % v/v), and pretreated OMSR with NaOH (0.5 %, 1.5 % w/v) and H_2SO_4 (0.5 %, 1.5 % v/v) whole biomass. It was shown that in all cases pre-treatment enhanced the consumption of carbohydrates as well as ethanol final yields.

KEYWORDS: bioethanol, solid wastes, OMW, xylose, yeasts.

1. INTRODUCTION

Among the so called clean fuels, ethanol is considered to be one of the most promising and is a good choice as a fuel additive. Moreover, ethanol made from lignocellulosic wastes provides economic as well as environmental advantages. This is due to the fact that the availability of huge quantities of residual lignocellulosic biomass makes the alcohol production process appealing not only in terms of commercial competition, but also in terms of sustainability (Parawira and Tekere, 2011). Significant advances have already been achieved at lab scale, concerning the exploitation of lignocellulosic materials. However, major issues still remain to be technical and economical obstacles and/or bottlenecks for upgrading the process to full scale. These issues involve among others, the pre-treatment of the biomass, which is necessary in order to facilitate the accessibility of cellulose and hemicellulose to microorganisms, thus enhancing the overall ethanol yield.

The production process of olive oil, one of the main agricultural products in the Mediterranean area, leads to the generation of large quantities of liquid and solid wastes. As shown from previous studies, these wastes can represent an environmental hazard when disposed directly to the

environment, due to their high organic load and toxic effect to microorganisms, plants (Azbar *et al*., 2004) and, as recently shown, to marine organisms (Danellakis *et al*., 2011). The type and quantity of such wastes depend on the type of extraction method used during the process. From the commonly used three-phase mills, two are the main streams of wastes that emerge, i.e. the liquid olive oil mill wastewater (OMW) and olive oil mill solid residues (OMSR). The latter consists of the remaining pulp of olive processing after the extraction of oil, as well as the cracked seed of the olive fruits, containing thus mainly lignocellulose and residual oil. The commonly used practice for OMSR management is combustion, after having extracted the residual oil by secondary extraction using organic solvents (Niaounakis and Halvadakis, 2006). Other proposed ways of OMSR management are their exploitation as substrate for edible fungi production and compost (Filippi *et al*., 2002; Vlyssides *et al*., 2008), and as feedstock for bio-fuels generation, such as methane (Boubaker and Cheikh, 2007) and bio-ethanol (Ballesteros *et al*., 2001). In the latter case, the complex carbohydrates (cellulose and hemicellulose) of the lignocellulose of OMSR have to be degraded towards their simple sugars and be further fermented via yeasts or bacteria. In order though for carbohydrates to be fully exploitable, their liberation from the lignin seal and their further hydrolysis has to be achieved, both of which can be facilitated via pre-treatment methods (Fan *et al*., 1981).

Yeasts traditionally used in alcoholic fermentation are able to consume hexoses as substrate but not pentoses. *Pachysolen tannophilus* consumes both C5 and C6 sugars (Kavanagh and Whittaker, 1994), fermenting them towards ethanol and xylitol, even under aerobic conditions (Sanchez *et al*., 2004). Actually, *P. tannophilus* was the first yeast identified as being capable of alcoholic fermentation of the abundant aldopentose D-xylose, that is derived from wood degradation (Schneider *et al*., 1981). Since then, a variety of yeasts have been shown to be capable of fermenting xylose, but still when compared to most of those, the fermentation properties of *P. tannophilus* appear quite inferior (DuPreez *et al.,* 1984; Delgenes *et al.,* 1986).

The present study aimed atthe investigation of the effect of thermochemical pre-treatment on the ethanol production yield from OMSR, using *P. tannophilus*. OMSR was subjected to acid and alkali treatment at 130^{o}C for 45 min. The thermal treatment profile used was previous proven to be optimum in terms of direct saccharification and subsequent enzymatic digestibility by previous experiments (Ntaikou *et al*., 2010). Nine different types of OMSR based substrates were tested i.e. untreated biomass, hydrolysates generated from pretreated OMSR with NaOH (0.5 % w/v, 1.5 % w/v) and H_2SO_4 (0.5 % v/v, 1.5 % v/v), and whole biomass of OMSR pretreated with NaOH (0.5 % w/v, 1.5 % w/v) and H_2SO_4 (0.5 % v/v, 1.5 % v/v). Subsequently, the substrates were subjected to simultaneous saccharification and fermentation (SSF) by adding cellulolytic enzymes to the fermentation media. The effect of the pre-treatment was evaluated in terms of the enhancement of carbohydrates consumption and maximum observed ethanol yields.

2. MATERIALS AND METHODS

2.1. Feedstock

Olive oil mill solid residue (OMSR) was obtained from a three-phase olive mill of Patras, Greece. The physicochemical characteristics of the waste are presented in Table 1. OMSR was collected immediately after the olive extraction process, and was stored in batches at -21 ^{o}C until use. Before use biomass was dried at 105^{o}C until stabilization of weight (~ 1day). The dried biomass was subjected to mechanical treatment, using a stainless steel grinder mill. For technical reasons, stones with diameter above 1mm were removed, thus resulting to a stones removal of ~25-30 %, corresponding to 9-15 % removal of initial total biomass.

2.2. Microorganism, media and growth conditions

All fermentation tests were performed with the yeast *P. tannophilus*, strain DSMZ 70352. The yeast was stored at 4^{0}C in slant solid cultures,using a medium with the following composition (in g l^{-1}): yeast extract 3; malt extract 3; peptone 5; D-xylose 5; D-glucose 5; agar–agar 20. For starting each experiment, the microorganism was inoculated under sterile conditions in test tubes, with10 ml of fresh liquid medium of the above described composition, and the cultures were incubated at 30 ^{0}C for 60 h with mechanical agitation of 100rpm, in order to obtain cells at the same growth stage. The cells were then harvested via centrifugation and used as inocula.

Table 1. Physicochemical characteristics of OMSR that was used in the present study

Parameter	Value
Humidity (%)	53.2 ± 1.4
Oil (%)[1]	11.8 ± 1.42
Phenols (mg g^{-1} biomass)[1]	13.0 ± 0.8
Total carbohydrates[*] (g g^{-1} biomass)[1]	0.49± 0.12
Soluble sugars (g g^{-1} biomass)[1]	0.02 ± 0.00
Ash (%)	>1.5
pH (suspension 5 % w/v)	4.9 ± 0.1

[1]on dry basis

[*]measured as glucose equivalents

2.3. Pre-treatment

Thermochemical pre-treatment was performed in order to facilitate the liberation of sugars from the cellulosic and hemicellulosic fraction of OMSR. Milled OMSR was suspended to aquatic solutions of H_2SO_4 and NaOH so as to correspond to 5 % initial solids loading (5 g per 100 ml of added liquid). Suspensions were then subjected to thermal treatment at 130^oC for 45 min. After cooling down, the suspensions were either filtered under vacuum using glass fiber filters (0.7 μm pore size), so as to recover the hydrolysates, or used as is. Prior to fermentation tests pH was in all cases adjusted to 4.8 with 4N NaOH or 4N HCl.

2.4. Fermentation tests

Two types of fermentation tests were performed, a) direct fermentation of substrates consisting from simple sugars i.e. commercial sugars and hydrolysates, and SSF (Simultaneous Saccharification and Fermentation) tests of more complex substrates containing simple sugars and/or lignocellulosic biomass, i.e. raw OMSR and pre-treated OMSR. In overall, nine different OMSR based substrates were used as carbon source: raw OMSR biomass without any chemical or thermal treatment, containing mainly complex carbohydrates; OMSR hydrolysates after thermochemical treatment with H_2SO_4 (0.5 %, 1.5 % v/v) and NaOH (0.5 %, 1.5 % w/v), containing simple sugars; OMSR after thermochemical treatment with H_2SO_4 (0.5 %, 1.5 % v/v) and NaOH (0.5 %, 1.5 % w/v), containing both simple sugars that were liberated during pre-treatment and compex carbohydrates that were not degraded. Experiments with commercial glucose and xylose were also conducted, so as to compare the behavior of the yeast in synthetic media. In all cases the media were supplemented with the following nutrients (in g l^{-1}): $MgSO_4$, 1; KH_2PO_4, 1; $(NH_4)_2SO_4$, 1; peptone, 1; yeast extract, 1.5. The final pH was adjusted again to 4.8 with 4N NaOH or 4N HCl when needed, and subsequently the media were sterilized at 121 ^0C for 20 min. All fermentation tests were performed in sterile 250 ml Erlenmeyer flasks at 30 ^{0} C and constant mechanical agitation of 100 rpm. Each Erlenmeyer flask contained 100 ml of medium. In the case of direct fermentations (hydrolysates, commercial sugars) direct inoculation of the media was performed using yeast cells that were harvested from 10 ml liquid cultures as described in section 2.2. In the case of SSF tests, and since *P. tannophilus* does not have fibrolytic properties, a mixture of cellulases and endoglucanases was added to the media prior to inoculation. The enzymes used were Celluclast 1.5 l (30 FPU g^{-1} initial solids) and Novozyme 188 (40 FPU g^{-1} initial solids), and they were added to the media under sterile conditions. After the addition of enzymes, the suspensions were incubated at 40^oC for 3 h, and subsequently were inoculated with yeast cells that were harvested from 10 ml liquid cultures as described above.

3. RESULTS AND DISCUSSION

Apart from hydrolysates and pre-treated OMSR biomass, pure glucose and xylose were also used as carbon sources for ethanol production. As shown in Figure 1, *P. tannophilus* seems to ferment glucose with a higher rate than xylose, leading also to a higher ethanol yield. Indeed the estimated ethanol yield from glucose fermentation was threefold higher than that from xylose (Table 2).

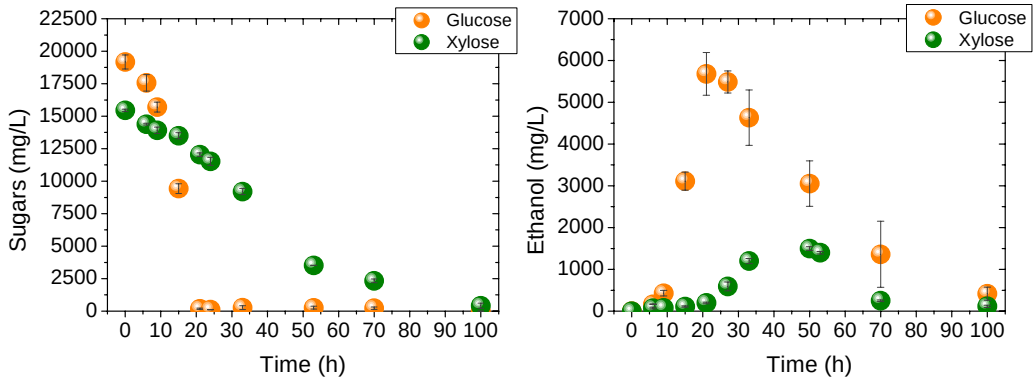

Figure 1. Consumption of sugars and ethanol production from glucose and xylose during fermentation with *P. tannophilus*

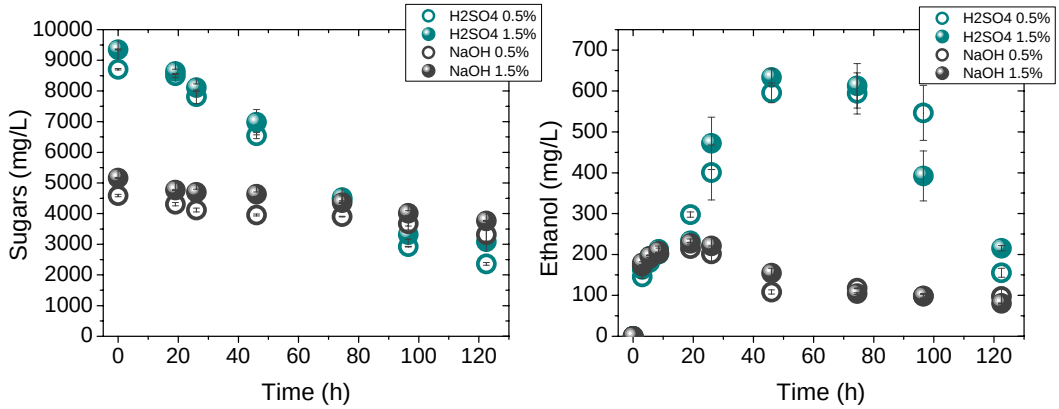

Figure 2. Consumption of sugars and ethanol production from OMSR hydrolysates during fermentation with *P. tannophilus*

In Figure 2 the consumption of sugars contained in OMSR hydrolysates, as well as ethanol production versus time are presented. As shown the hydrolysates generated from the pre-treatment of OMSR were of different initial sugars concentrations. Acid pre-treatment resulted to hydrolysates of 8.71 ± 0.02 g l^{-1} total sugars, measured as glucose equivalents and 9.35 ± 0.01 g l^{-1} total sugars, for H_2SO_4 0.5 % v/v and H_2SO_4 1.5 % v/v, respectively. Alkali pre-treatment led to much lower saccharification, resulting thus to hydrolysates of 4.59 ± 0.04 g l^{-1} total sugars and 5.16 ± 0.01 g l^{-1} total sugars for NaOH 0.5 % w/v and NaOH 1.5 % w/v, respectively. Such differences on the saccharification degree were expected, since during acid pre-treatment, hemicelluloses are mainly attacked and are almost fully solubilised, leading to the liberation of pentoses (Nigam, 2002; Roberto *et al*., 2003), whereas during alkaline pre-treatment, lignin is mainly attacked and carbohydrates are solubilised to a lower degree (Balaban and Ucar, 1999). The above are also in agreement with the findings of the present study since, as shown in Table 2, the ethanol yield, estimated in terms of sugars' consumption from alkali hydrolysates, is almost double compared to that from acid hydrolysates.

Table 2. Consumption of sugars and ethanol yields during fermentation of pure substrates and hydrolysates with *P. tannophilus*

Carbon source	**Sugars uptake** (%)	**Ethanol $_{max}$ yield** (g eth g^{-1} sugar)	**Ethanol $_{max}$ yield** (ml eth kg^{-1} OMSR[1])
Glucose	100 ± 0.00	0.30 ± 0.01	-
Xylose	100 ± 0.00	0.10 ± 0.01	-
Hydrolysate, H_2SO_4 0.5 %	72.88 ± 0.49	0.09 ± 0.00	15.10 ± 0.71
Hydrolysate, H_2SO_4 1.5 %	67.02 ± 0.25	0.10 ± 0.01	16.05 ± 1.54
Hydrolysate, NaOH 0.5 %	27.75 ± 4.65	0.17 ± 0.01	5.46 ± 0.32
Hydrolysate, NaOH 1.5 %	27.06 ± 0.07	0.16 ± 0.01	5.77 ± 0.27

[1]on dry basis

However, when ethanol is estimated in terms of whole biomass of OMSR the results are reversed, thus leading to a threefold decrease for alkali pre-treatment. This was attributed to the lower sugars liberation, but also to the fact that sugars' consumption was also lower for alkali hydrolysates (~30 %) than for acid hydrolysates (~70 %). It also has to be mentioned that the concentration of additive, during pre-treatment, did not have any significant effect on the results. In figures 3 and 4, the consumption of total and soluble carbohydrates and the production of ethanol are illustrated, during simultaneous saccharification and fermentation of raw and pre-treated OMSR biomass, respectively.

Regarding the consumption of carbohydrates, similar behavior was observed for all types of pre-treated biomass. Thus, the consumption of either total or soluble carbohydrates had similar rates, being in all cases much slower than when raw OMSR was used. Indeed, in 33 h, carbohydrates' consumption for raw OMSR ceased and maximum ethanol production was achieved, whereas in all cases of pretreated biomass, the uptake of carbohydrates ceased at about 90h of fermentation. In the latter cases, maximum ethanol production was observed before 90 h, indicating that after a certain point, microbial growth continues on the expense of ethanol, even when carbohydrates are still available. This could be attributed to the rather high aeration level. It is indeed reported that aeration plays a crucial role in stimulating the fermentation of sugars by *P. tannophilus* (Du Preez *et al*., 1984), thus being necessary for the experiment. However, thoroughly aerobic conditions can lead to biomass accumulation by consumption of the fermentation products (Watson *et al*., 1984).

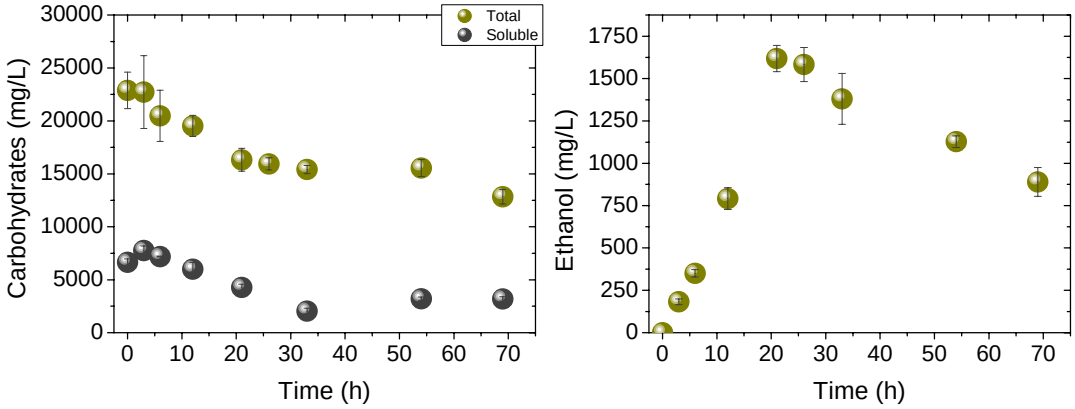

Figure 3. Consumption of total and soluble carbohydrates and ethanol production from raw OMSR during SSF with *P. tannophilus*

As shown in Table 3, it can be assumed that pre-treatment of biomass facilitated considerably the biotransformation of carbohydrates, since it not only resulted to higher carbohydrates' removal, but also led to higher ethanol yields. It is observed that in terms of total carbohydrates' consumption, alkali treatment gave the best results. Moreover, treatment with higher concentration of chemicals improved slightly sugars' reduction for both alkali and acid pre-treatment.

Table 3. Consumption of carbohydrates and ethanol yields during fermentation of raw and pre-treated OMSR with *P. tannophilus*

Carbon source	**Carbohydretes uptake** (%)	**Ethanol $_{max}$ yield** (g eth g^{-1} carb.)	**Ethanol $_{max}$ yield** (ml eth kg^{-1} OMSR[1])
Raw OMSR	32.01 ± 2.12	0.16 ± 0.01	28.12 ± 4.13
OMSR, H_2SO_4 0.5 %	48.44 ± 3.74	0.30 ± 0.02	44.90 ± 3.02
OMSR, H_2SO_4 1.5 %	50.35 ± 0.88	0.28 ± 0.01	49.59 ± 1.79
OMSR, NaOH 0.5 %	51.00 ± 4.98	0.24 ± 0.02	35.71 ± 2.02
OMSR, NaOH 1.5 %	63.46 ± 5.17	0.20 ± 0.03	41.43 ± 1.33

[1]on dry basis

In terms of ethanol production, acid pre-treatment seemed to facilitate alcoholic fermentation, with 0.5 % H_2SO_4 resulting to the highest yield, measured either as g ethanol per g of consumed carbohydrates or as ml ethanol per kg of biomass. More specifically, pre-treatment with 0.5 % H_2SO_4 led to 87 % and 40 % increase of ethanol yield, measured as g ethanol per g of carbohydrates

and l ethanol per kg of OMSR, respectively. Pre-treatment with 1.5 % H_2SO_4 led to 76 % (gE g^{-1} S) and 35 % (ml E/ kg^{-1} OMSR) increase of ethanol yields, whereas 0.5 % and 1.5 % NaOH led to 51 % (gE g^{-1} S) and 23% (ml E kg^{-1} OMSR), and 25 % (gE g^{-1} S) and 11 % (ml E kg^{-1} OMSR) increase of ethanol yields, compared to the yields obtained from raw biomass fermentation. As also shown by the results of Table 3, the concentration of different chemical additives, affects positively the liberation of sugars from lignocellulosic biomass, as well as their subsequent biotransformation towards ethanol.

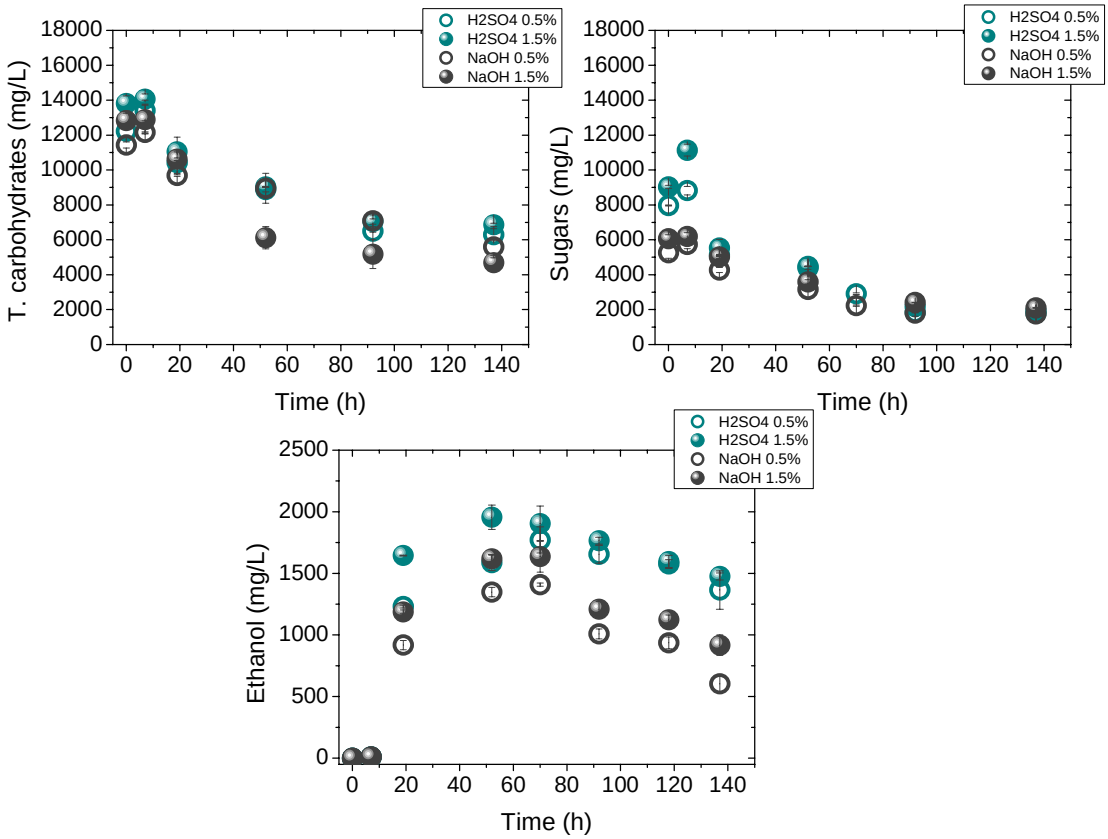

Figure 4. Consumption of total and soluble carbohydrates and ethanol production from thermochemically pre-treated OMSR during SSF with *P. tannophilus*

4. CONCLUSIONS

P. tanophilus is a C5 and C6 consuming yeast that is considered to be a good candidate for ethanol production from lignocellulosic biomass. Since, however, it does not have fibrolytic properties, the exploitation of such biomass can be achieved only after chemical or enzymatic saccharification. In the present study, the effect of thermochemical pre-treatment on ethanol production from OMSR was investigated. OMSR biomass was subjected to thermochemical pre-treatment with NaOH (0.5 % w/v and 1.5 % w/v) and H_2SO_4 (0.5 % v/v and 1.5 % v/v). After pre-treatment, either the hydrolysates which were rich in readily fermented sugars, or the whole biomass supplemented with cellulolytic enzymes were used for ethanol production. It was shown that sugars' consumption was higher for hydrolysates from acid pre-treatment. Moreover, those hydrolysates led to better ethanol yields that the ones from alkali pre-treatment, for both concentrations of chemical agents used. However, even higher yields were obtained when whole OMSR pretreated biomass was fermented via SSF. In the latter case, ethanol yields, measured as l of produced ethanol per kg of dry OMSR biomass, increased for both acid and alkali pre-treatment. The highest yield was obtained for pre-treated biomass with 1.5 % H_2SO_4 and was 49.59 ± 1.79 ml ethanol per kg of OMSR.

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