

Saccharification of Delignified Rice Straw by *Fusarium oxysporum* for Ethanol Production by *Saccharomyces cerevisiae*

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IN THE PRESENT work, carrying out consolidated bioprocessing by mixed cultures of cellulytic fungus and yeast capable of fermenting hexoses was under scope. A preliminary test proved that there was no contradiction between using *Saccharomyces cerevisiae* as single culture or mixed with *Fusarium oxysporum* in fermenting glucose to ethanol. Rice Straw was subjected to delignification by alkaline/hypochlorite method. Delignified rice straw (RS) and carboxy methyl cellulose (CMC) individually were used in bioconversion process for ethanol production. *Fusarium oxysporum* performed fermentation with and without aeration, achieving ethanol production of 0.8 μ l/ml and 2.9 μ l/ml, respectively, proving the importance of air absence in this process. The CMC and RS were applied in a submerged culture to give maximum cellulases activity of 0.444U and 1.474U after 8 days, respectively. In order to apply consolidated bioprocessing, saccharification of either CMC or RS by *F. oxysporum* was applied in a single submerged culture giving maximum saccharification efficiency of 16.8% and 16% after 7 and 11 days, respectively. In the same submerged culture, *Saccharomyces cerevisiae* was used to ferment the released sugar under no aeration achieving ethanol production of 6.1% and 3.878% (v/v) corresponding to fermentation efficiency of 42.4% and 34.4%, respectively.

Keywords: Saccharification, Ethanol fermentation, Rice straw, CMC, *Fusarium oxysporum*, *Saccharomyces cerevisiae*.

Introduction

The key objective in any alcohol fermentation process is to reduce energy requirements and production costs. Cellulases production is an important step preceding production of ethanol in an economic process using any renewable cellulosic wastes (Mazharuddin khan *et al.*, 2011). Rice (*Oryza sativa*) straw as an abundant agro-residue, has an approximate annual production of 731 million tons produced worldwide, from which around 205 billion liters of bio-ethanol / year can be produced (Narra *et al.*, 2012). Rice straw contains approximately 30–35% cellulose, 25–30% hemicellulose, 15–28% lignin, and 4–7% ashes. Cellulose and hemicellulose from these lingo-cellulosic materials are potent precursors for the second generation of biofuel. However, chemical fractionation and biological manipulation of these lingo-cellulosic materials are limited due to their complex matrix structure including

their lignin content, acetylation and crystallinity (Sakdaronnarong and Jertjunya, 2012).

Therefore, an effective pretreatment for rice straw is required to remove lignin to efficiently increase cellulases access to cellulose. Beside acid and oxidative reagents, alkali pretreatment appears to be the most effective in breaking the ester bonds between lignin, hemicellulose and cellulose, as well as avoiding fragmentation of the hemicelluloses polymers (Narra *et al.*, 2012). In this regard, Mohy *et al.* (2015) considered alkaline oxidation pretreatment as the most effective, simple and economic pretreatment. On this regard, they found that one-half of lignin and most of hemicellulose content in rice straw was solubilized by alkaline hypochlorite pretreatment at room temperature after 24h. Added to that, crystallinity index decreased and consequently cellulose content increased in the pretreated biomass. After pretreatment, ethanol production requires different steps of hydrolysis (saccharification) and

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efficient ethanol recovery. Hydrolysis of biomass is essential for generation of fermentable sugars which are then converted to ethanol by microbial action (Sukumaran *et al.*, 2009).

The degradation of cellulose to soluble sugars requires a cooperative action of number of enzymes together: endo-glucanases (EC 3.2.1.41), exo-glucanases (EC3.2.1.91) and β -glucosidases, collectively known as cellulases. Unlike cellulose (a linear glucose polymer), hemicelluloses are hetero polymers of a range of pentoses primarily xylose and arabinose, as well as hexoses such as galactose, mannose, other sugars and their uronic acids (Okeke and Obi, 1995; Sukumaran *et al.*, 2009). Cellulase is produced via two methods: submerged liquid process and solid state process. Besides, most previous researches conducted cellulose hydrolysis in submerged process with many advantages regarding the process control (Shaibani *et al.*, 2012). *F. oxysporum* probably more commonly known as a phyto-pathogen has the ability to produce a wide range of biomass degrading enzymes. It was found that *F. oxysporum* can generally use both hexoses and pentoses with the ability to ferment them to ethanol as had been discovered before (Christakopoulos *et al.*, 1989; Singh *et al.*, 1991; Zhou *et al.*, 2002).

Hydrolysis and fermentation can be separately or simultaneously performed. In "Separate Hydrolysis and Fermentation" (SHF); hydrolysis and fermentation are applied sequentially in different bioreactors, while in "Simultaneous Saccharification and Fermentation" (SSF); cellulose hydrolysis and hexose fermentation are applied simultaneously in the same bioreactor (Shaibani *et al.*, 2012). Anasontzis and Christakopoulos (2014) stated that *F. oxysporum* was able to ferment monosaccharides to ethanol under anaerobic or micro-aerobic conditions. It bypasses the yeast by its ability to ferment pentoses, while yeast can ferment hexoses faster and better than fungi. In order to make *F. oxysporum* become advantageous in a realistic sense, a number of improvements must be done. This was why the present study was conducted to use this fungus individually in both cellulase production and saccharification of pretreated rice straw (RS), while studying its role in ethanol production with/without *S. cerevisiae* in a simultaneous action.

Materials and Methods

Rice straw was collected from a local farm in El-Sharkia Governorate and cut into small fragments,

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washed thoroughly and dried under vacuum at 60°C for 2 days. Pretreatments of rice straw by alkaline hypochlorite were carried out as described by Mohy *et al.* (2015). Ten grams of chopped rice straw were suspended in 200 ml of 1.5% NaClO at pH 11.5 (0.3 g NaClO /g substrate) in a 500 ml-conical flask, shaken overnight (100 rpm) at room temperature. Pretreated rice straw (RS) was filtrated and neutralized to pH 6.5-7.0 by 1N HCl, then thoroughly washed with distilled water and dried. *F. oxysporum* and *S. cerevisiae* were generously offered by Department of Microbiology, Soil, Water and Environment Research Institute. Starter cultures of *F. oxysporum* in PDA broth (Domsch *et al.*, 1980) and *S. cerevisiae* culture in YM broth (Difco, 1985) were used.

On the other hand, modified Mandels medium was the basal medium used for enzyme production (Patel *et al.*, 2007) in which 1% carboxy methyl cellulose (CMC) or RS added as carbon sources and inoculated by *F. oxysporum* pre-culture (5% v/v). The growth medium pH was adjusted at 5, incubated at room temperature for required days under test on a rotary shaker at 125 rpm. The filtered broth was collected for determination of enzymatic activities (Roy *et al.*, 1993). The enzymatic saccharification was started at 50°C in the same enzyme production broth for the required time under test.

The cellulases activity was estimated in terms of filter paper cellulases activity (FPase) using filter paper (Whatman, No.1) according to Ghose (1987). Glucose from produced after FPase action was measured with 3,5-dinitrosalicylic acid reagent, according to Miller (1959). One unit of FPase activity (U) was defined as the amount of enzyme that can release 1 mg reducing sugar as glucose per 1hr, under assay conditions, in 1ml reaction mixture. Saccharification efficiency % was calculated according to Gould (1984). Pure glucose was used as the sole carbon source at a concentration of 10% (w/v) in the modified Mandels medium in narrow necked bottles for testing fermentation by single and mixed cultures of *F. oxysporum* and *S. cerevisiae*. Bottles were kept stagnant during fermentation at room temperature.

Cotton plugged bottles were filled to half of its volume with Mandel's medium with either RS or CMC and were shaken at 120 rpm then incubated at 35°C to ensure cellulases production by the fungal inoculum during a saccharification period of 2 days. The same bottles were completed up to three fourths of their volumes with sterile

fermentation medium to minimize air volume. In such fermentation tests, two methods were applied to control aeration conditions. Bottles were either cotton plugged (aeration) or firmly capped (no aeration) with stoppers and in both cases the same individual fungi, which was responsible for saccharification allowed to ferment the released sugars.

Spectrophotometric determination of ethanol was carried out according to Caputi *et al.* (1968), where ethanol was expressed as μl ethanol /ml. The Fermentation medium based on the modified medium recommended by Sarabana *et al.* (2015) was employed. Starter cultures from *F. oxysporum* and *S. cerevisiae* were adjusted to a fixed dry biomass% (w/v) and used for inoculation of the fermentation medium so that the inoculant volume didn't exceed 10% v/v (Kenealy and Dietrich, 2004). The fermentation medium volume was either 100 ml in 250 ml conical flasks to ensure best aeration, or 150ml enclosed in 200ml firmly closed bottles to maintain minimized air condition for fermentation process. Saccharified RS or CMC by fungi was introduced to fermentation by either yeast or fungi without sterilization to fulfill consolidated bioprocessing fundamental.

Calculations (Mohy *et al.*, 2015):

$$\text{Saccharification degree (\%)} = \frac{R(\text{mg}) \times 100}{C(\text{mg}) \times 0.9}$$

$$\text{Ethanol fermentation efficiency \%} = \frac{\text{ethanol actual yield} \times 100}{\text{ethanol theoretical yield}}$$

where; Ethanol theoretical yield % from hexoses = 51.2%.

where; R: total released reducing sugar, C: Total carbohydrates in the substrate (RS)

Data were statistically analyzed for the least significant differences (LSD) adopting the procedure mentioned by Gomez and Gomez (1984).

Results and Discussion

Fermentative performance in single or mixed cultures by *F. oxysporum* and *S. cerevisiae*

The performance of yeast under minimum aeration wasn't affected when the yeast strain was grown in pure or in a mixed culture, giving 8.867 $\mu\text{l}/\text{ml}$ and 7.870 $\mu\text{l}/\text{ml}$, respectively (Figure 1). In both cultures, maximum ethanol production after 12 hour ranged from 7.8 to 8.9 $\mu\text{l}/\text{ml}$, statistically considered convergent performance, indicating the absence of antagonism between the two strains. Growing *F. oxysporum* in a pure single culture resulted in lower ethanol production with a maximum production of 3.7 $\mu\text{l}/\text{ml}$ after 48 hours. The actual fermentation efficiency by yeast in a mixed or single culture achieved nearly 12.2 to 13.7% of the theoretical efficiency in the first 12 hours. Depending on this finding, the unfermented glucose mostly played as a carbon source for yeast metabolism.

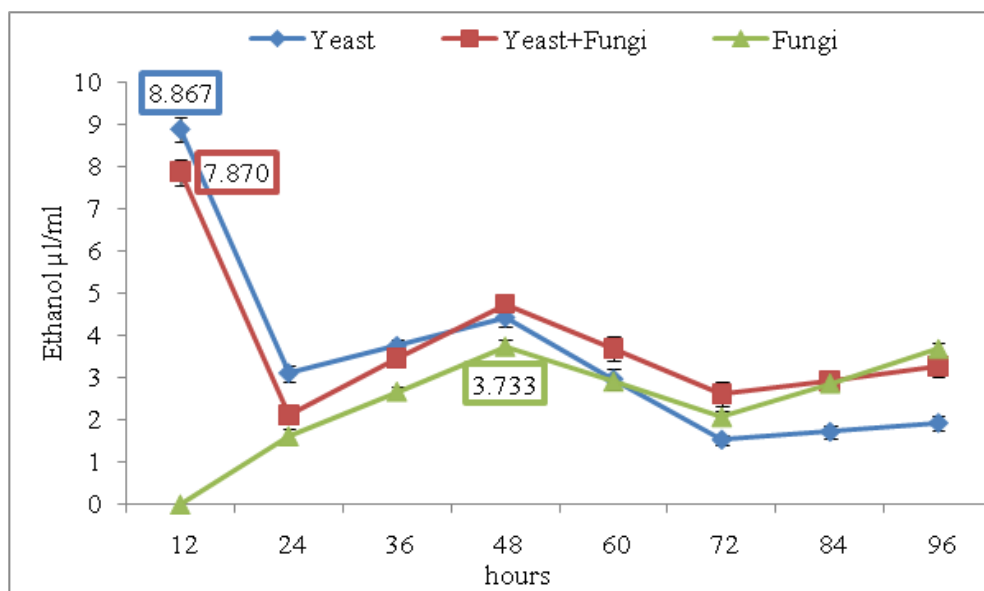


Fig. 1. Ethanol production from 10% glucose by *F. oxysporum* and *S. cerevisiae*, either in pure or as mixed cultures, under minimum aeration (LSD= 0.322).

Previous reports by Dashko *et al.* (2014) referred to the ability of the yeast *Saccharomyces cerevisiae* to thrive and ferment sugars under either aerobic or anaerobic conditions. However, the yeast performance varies according to aeration magnitude which emphasizes in the present work the drop in fermentation efficiency down to 13.5% than in ideal anaerobic conditions. Alam *et al.* (2007) tested the possibility of ethanol production in presence of both cellulolytic fungi and fermentative yeast. They found that combination of *S. cerevisiae*, *Phanerochaete chrysosporium*, *Trichoderma harzianum* was compatible and able to produce ethanol through utilizing rice straw in a direct bioconversion process with high efficiency than single cultures. This finding meets with the absence of antagonism between *S. cerevisiae* and *F. oxysporum* in the present work.

Effect of aeration on ethanol production by F. oxysporum grown on CMC and RS

F. oxysporum was grown on either CMC or RS at 35°C during the first two days to encourage cellulases production and saccharification of either substrates, after which the fermentation process began under controlled conditions as suggested before, through which ethanol was produced as shown in figures (2; a and b). The results suggested that turning aeration conditions to its minimum pushed the fungi to ferment available fermentable sugars parallel to a prolonged cellulolytic action and glucose production from the substrate during 13 day fermentation period. In

such a mixed process, ethanol quantity increased cumulatively during almost all the fermentation periods, which dropped after words which might be due to ethanol consumption by the fungus itself as suggested by El-Gabry *et al.* (2016).

On the other hand, the CMC mostly induced endo-cellulases production in the first 2 days with less efficiency in releasing reducing sugars if compared with the RS which mostly induced production of all cellulase types (endo-cellulases, exo-cellulases and β -glucosidases) due to their collective synergistic effect (Mohy, 2017). In spite of many possible inhibitory compounds including phenolic compounds and furan aldehydes that might have been formed during alkaline oxidative pretreatment of rice straw (Jönsson, and Martin, 2016) but there was no retardation in ethanol maximum production than with CMC. These findings affected indirectly the reducing sugar available for fermentation. Ethanol produced from saccharified RS was more than that when saccharified CMC was used, as in the aerated and non-aerated conditions the fungi gave 1.4 μ l/ml and 2.85 μ l/ml with RS, respectively. This might be due to the fungal ability to ferment pentoses released from saccharified RS after finishing fermentation of hexoses in the same source (Anasontzis and Christakopoulos, 2014). That was obvious from the semi stable level of ethanol produced during non-aerated fermentation of both saccharified CMC and RS after 6 days.

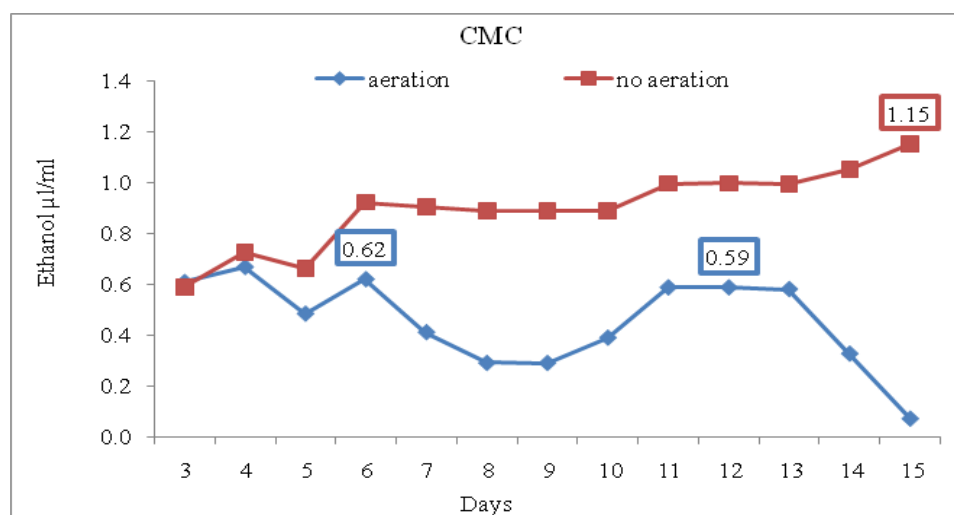


Fig. 2a. Ethanol production by *F. oxysporum* on saccharified CMC under aeration and no aeration conditions(LSD= 0.096).

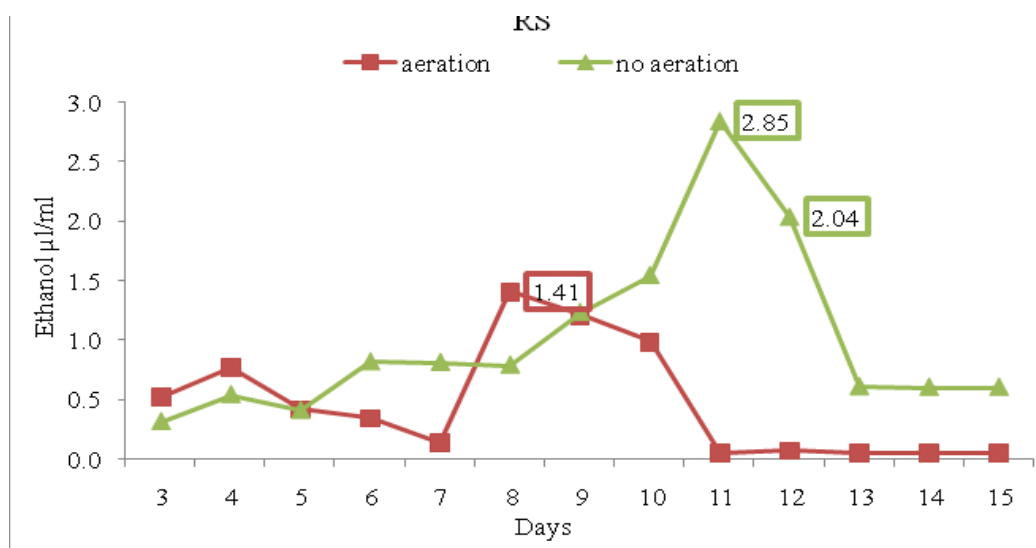


Fig.2 b. Ethanol production by *F. oxysporum* on saccharified RS under aeration and no aeration conditions (LSD 0.05 = 0.234).

Ethanol production from saccharified CMC and RS in single and mixed cultures of F. oxysporum and S. cerevisiae under no aeration

It appeared clearly that in case of CMC, the fungi responsible for producing cellulases used for saccharifying the CMC was negatively affected by saccharification temperature at 50°C (Fig. 3; a and b) which was obvious when additional fungal inoculum was added leading to elevated fermentation capability. This correlation was reversed in case of RS, as increasing fungal inoculum might had consumed sugars from saccharified RS in growth more than in fermentation and ethanol production, as it was previously noted by Sarbana *et al.* (2015) and El-Gabry *et al.* (2016). As it was explained that the presence of yeast extract as nitrogen source served as electron acceptor that compensate the scarcity of oxygen in minimum aeration leading to its growth over the expense of fermenting sugar to ethanol. Besides, this lights out the importance of fungal inoculum size ratio to available fermentable sugars. As this ratio increased, sugar portion mainly consumed in growth and vice versa. Moreover, the fungal cells responsible for cellulase production that was subjected to higher temperature (50°C) during saccharification was pushed to form more spores, which in turn regenerated faster with possibly more vital conditions at 35°C than the vegetative form as stated before by Patel *et al.* (2007), and in turn fermenting the sugars more efficiently, specially any possible pentoses, at day 3.

With CMC the fungal existence alone during the 5 days of fermentation allowed two parallel processes to take place. Mainly fermenting sugar resulted from previous saccharification process and very possible prolonged cellulytic action (mostly endo β 1,4 glucanase) and saccharification under 35°C allowing increase in sugar content leading to final increase in ethanol up to 2ml/ml. The existence of yeast with fungi took advantage of this phenomenon as ethanol produced was much than that by yeast alone.

Successive consolidated bioprocessing for ethanol production by fungi

This experiment was classified into three stages. At the first stage, *F.oxysporum* was grown at 35°C for 13 days on either CMC or RS (10%) as a sole carbon source for cellulases production. FPase was measured through the 13 days with occasional addition of N source every 5 days. Highest cellulases activity was 0.444 U and 1.474 U, achieved at 8th when the fungus was grown on the CMC and RS, respectively. Depending on those results, the previous experiment was repeated up till the 8th day to ensure maximum cellulases production on 35°C, after which the temperature was shifted up to be 50°C for applying efficient saccharification up till the 11th day. Worthy to mention that cellulases production was done distributed in 33 fermentation bottles to ensure sampling every day in triplicate individual bottles for either CMC or RS. The maximum released sugar measured was at the 7th and 11th days when the fungus was grown on CMC and RS recording

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1.44% and 1.13% (w/v), nearly corresponding to saccharification degree of 15.98% and 16.74%, as shown in Figures (4-b) and (5-b), respectively. The very slow increase in release of reducing sugars of saccharification may be primarily due to end-product inhibition and to some extent thermal instability during the long reaction period, as mentioned before by Okeke and Obi (1995).

All saccharified CMC and RS bottles were consequently inoculated with *S. cerevisiae* and more fermentation medium was added to minimize void volume for less aeration. The highest ethanol

production was achieved at the 7th and the 11th day, recording 0.605% and 0.388% (v/v), for CMC and RS, corresponding to fermentation efficiency of 95.6 and 78.2%, as shown in Figures (4-c) and (4-c), respectively. Compared to the work of Sukomara *et al.* (2009), they found that highest yield of reducing sugars was 26.3 g/L obtained from enzymatic hydrolysate of rice straw while in the present work it reached 11.3g/L. When they used it as substrate for ethanol production by *S. cerevisiae*, the ethanol yield was 0.09 g/ g pretreated rice straw while it was nearly 0.05 g/ g pretreated rice straw in the present work.

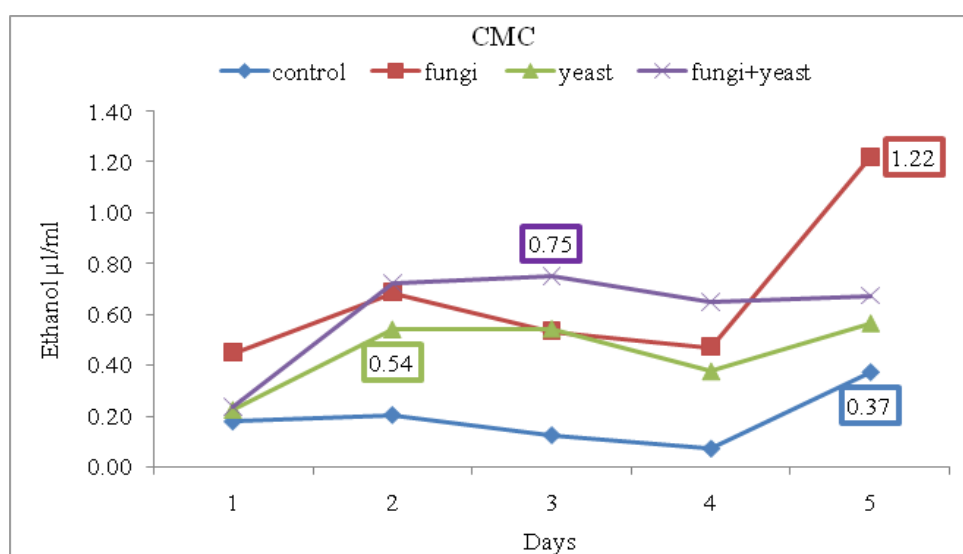


Fig. 3a. Ethanol production from saccharified CMC by *F. oxysporum* and *S. cerevisiae* inoculums applied individually or as mixed culture under no aeration (LSD 0.05 = 0.116).

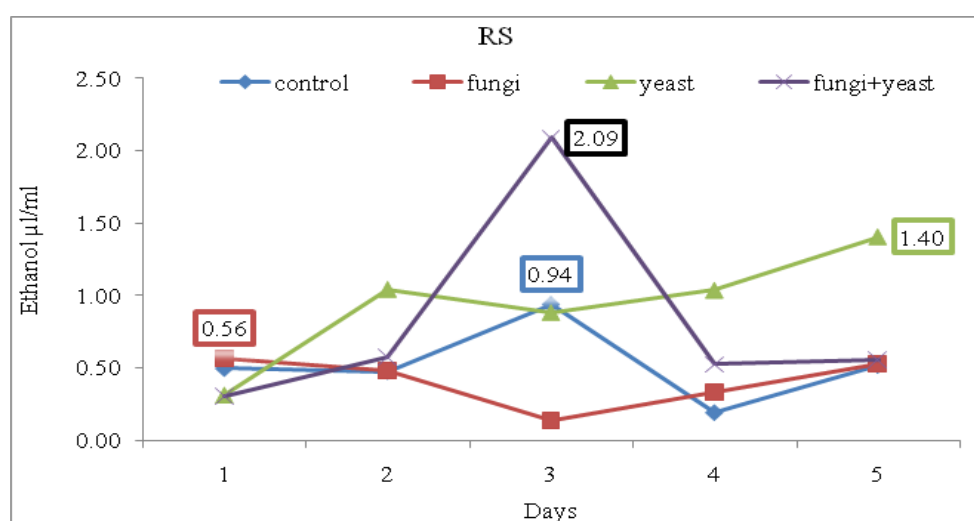


Fig. 3b. Ethanol production from saccharified RS by *F. oxysporum* and *S. cerevisiae* inoculums applied individually or as mixed culture under no aeration (LSD 0.05 = 0.256).

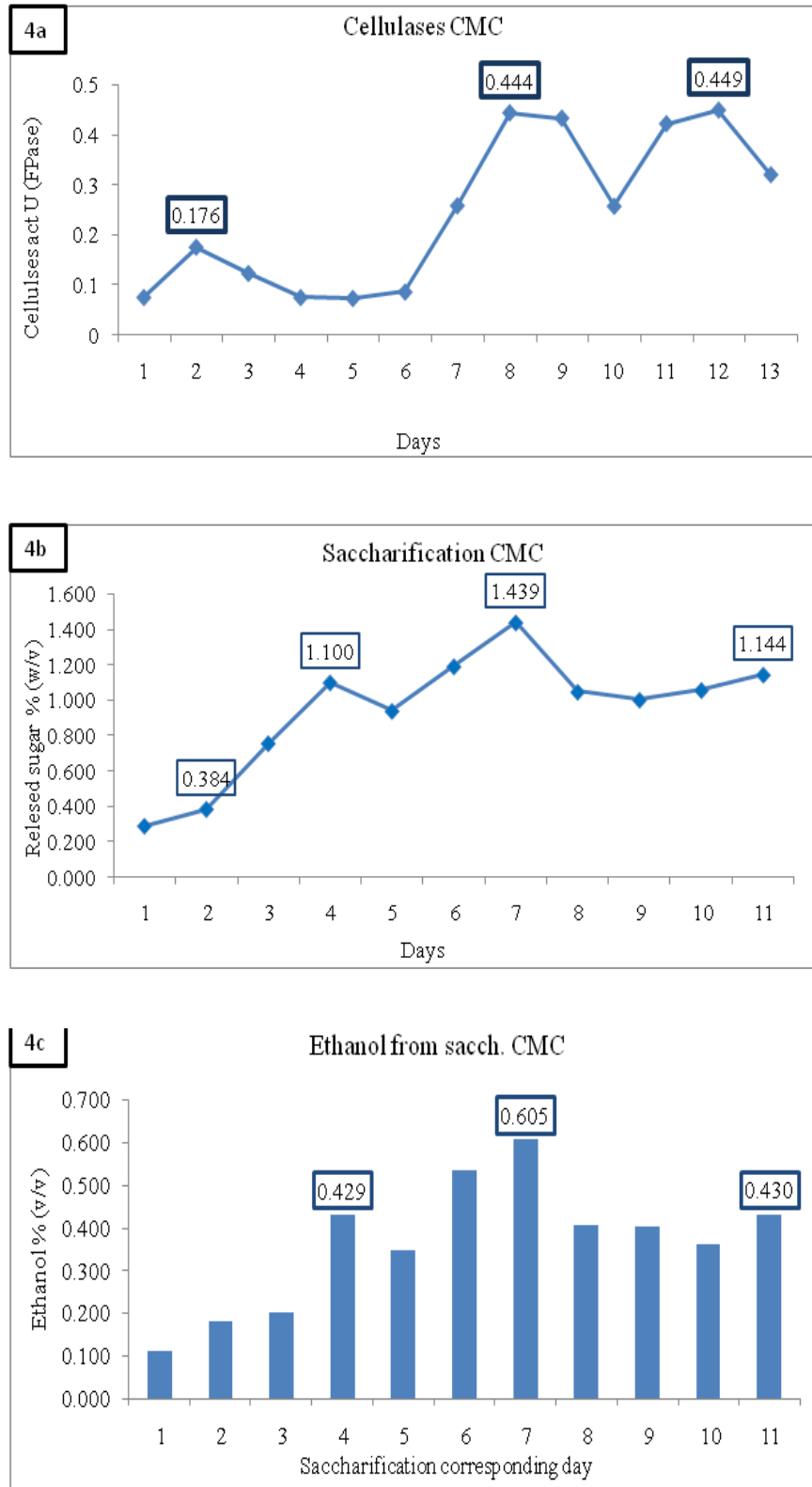


Fig. 4. a- Cellulases production (LSD 0.05 = 0.138), b- Saccharification of CMC (LSD 0.05 = 11.99) and c- Ethanol production on saccharified CMC under minimum aeration condition (LSD 0.05 = 0.052) by *F. oxysporum*.

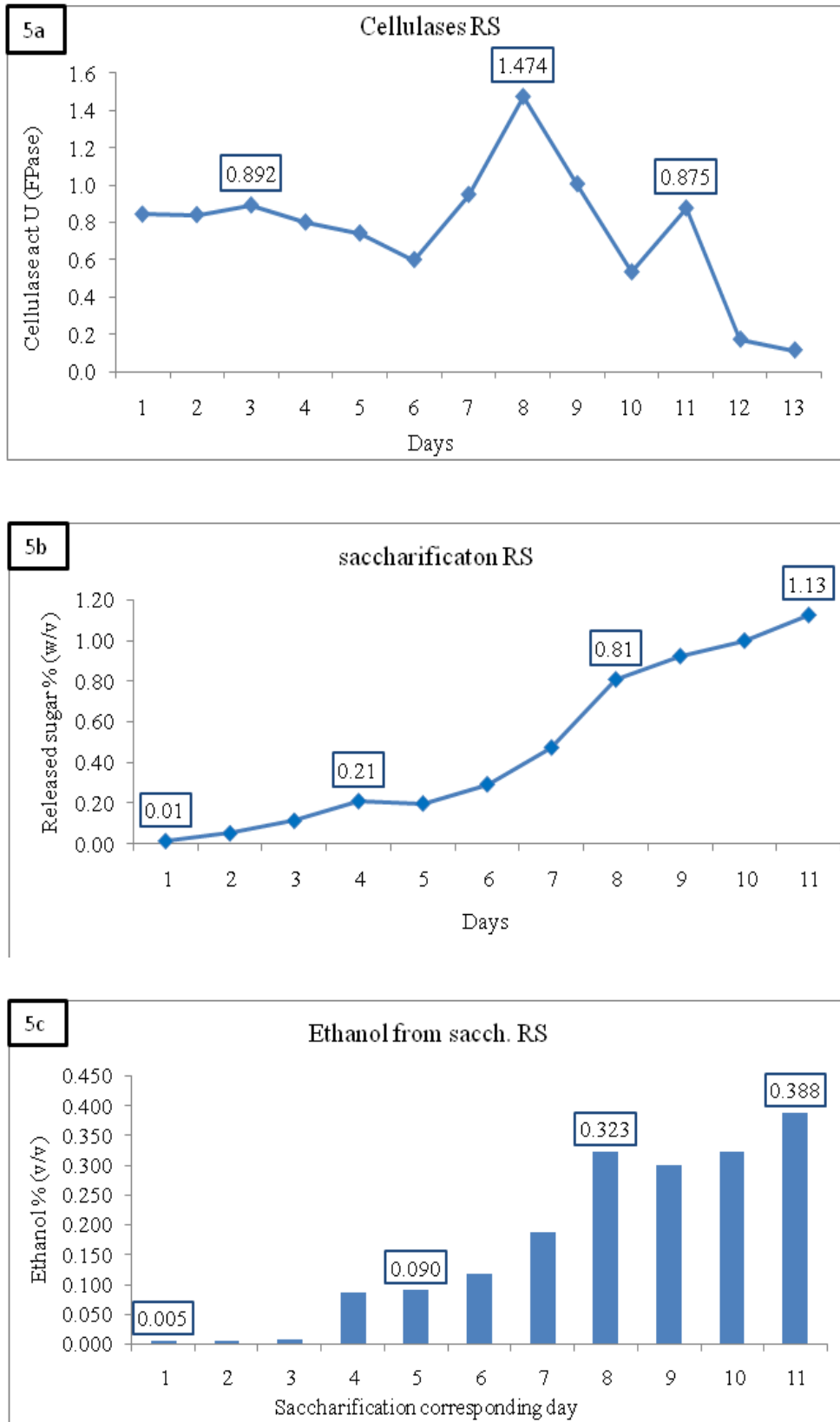


Fig. 5. a- Cellulases production (LSD 0.05 = 0.33), b- Saccharification of RS (LSD 0.05 = 8.133) and c- Ethanol production on saccharified RS under minimum aeration condition(LSD 0.05 = 0.34)by *F. oxysporum*.

On the other hand, Vintila *et al.* (2009) used cellulases produced by *Trichoderma viride* CMIT3.5 together with *S. cerevisiae* CMIT2.18 in applied simultaneous hydrolysis and fermentation of lignocellulose at the temperature of 40°C. This system can lower the price of ethanol produced from lingo cellulosic biomass. Also, Vintilai *et al.* (2011) found that the yeasts and cellulases can work in the same medium and at common parameters. Although common yeasts are able to ferment glucose and produce ethanol at 30 – 35°C and cellulases have the optimal activity at 50°C, certain yeast strains and cellulases can act together at common temperatures of 37 – 40°C and in the same medium. Then the previously mentioned *S. cerevisiae* CMIT2.18 can be successfully used to ferment glucose produced by cellulases in the same medium, at temperatures of 37 – 40°C in simultaneous saccharification and fermentation (SSF) process to convert lingo cellulosic biomass to ethanol.

Pasha *et al.* (2012) studied sequential cellulase production, saccharification and ethanol production experiment in a set, where *Aspergillus niger* which had been responsible for cellulase production was inactivated by autoclaving after saccharification. They found that the maximum fermentation efficiency by yeast was observed as maximum of sugars were utilized for ethanol production. Whereas in non-autoclaved set after saccharification, less fermentation efficiency was noted, as some of released sugars must have been utilized by *A. niger*, hence ethanol conversion by yeast was reduced. This observation was crucial in explaining the low fermentation efficiency of 78.2% compared to the theoretical efficiency.

Conclusion

From the foregoing results, it could be concluded that *F. oxysporum* achievement with CMC was more successful than with RS as it released more glucose during saccharification. It might be correlated to the type of substrate that induced the *F. oxysporum* to release the corresponding type of cellulases, as CMC is well known as a specific substrate and inducer for β 1,4 endoglucanase. On the other hand, RS that needs more cellulases types including endo and exocellulases beside β - glucosidases. Add to that, the complex and crystalline structure of cellulose forming RS than that in CMC beside the presence of residual lignin and ash acting as inhibitors (Mohkami and Talaeipour, 2011; Mohy *et al.*, 2015). Never the less, fermentation efficiency of sugar released from saccharified RS and CMC by

S. cerevisiae in presence of *F. oxysporum* wasn't relevant to glucose quantity, as saccharified RS negatively affected *S. cerevisiae* performance that might be correlated to the presence of residual lignin in RS predicted to be 8% and ash too (Mohy *et al.*, 2015), while it was absent in CMC. According to the obtained results, the present study established the possibility of using *F. oxysporum* on pretreated RS for the production of fermentable sugars with acceptable efficiency which can be further utilized for production of biofuel and other valuable commodities via industrial fermentation.

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