Middle East Journal of Agriculture Research
ISSN 2077-4605

Volume : 06 | Issue : 04 | Oct.-Dec. | 2017
Pages:1417-1425

Rice straw processing using *Phanerochaete chrysosporium* for cellulases production and enzymatic saccharification

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Received: 16 Oct. 2017 / Accepted: 20 Dec. 2017 / Publication date: 30 Dec. 2017

ABSTRACT

Phanerochaete chrysosporium was used for cellulases production and saccharification of rice straw (RS) and bagasse (Bag). A comparison was carried out between cellulases produced by *P. chrysosporium,* on RS pretreated with sodium hypochlorite (1.5% NaClO) (RS_1) or sodium hydroxide (2 % NaOH) (RS_2) and on bagasse untreated (Bag_1) or pretreated with 2% NaOH (Bag_2) in submerged processes. Results revealed that the highest FPase activity was achieved on RS_1 pretreated with NaClO on the 5th day (24.14 U). Compared to carboxymethyl cellulose (CMC) as a control, cellulases with highest Fpase activities were produced on the 5th and 8th day on RS_1 and CMC giving 27.67 U and 24.47 U, respectively. Furthermore, by applying sequential saccharification after cellulases production in the same container, 50.4% and 39.12% of RS_1 and CMC were saccharified on the 5th and the 3rd day, respectively. On the other hand, successive cellulases production on RS_1 and CMC and their utilization to saccharify new RS_1, the degree of saccharification was 49.7% and 38%, respectively.

Key words: Cellulases production, saccharification, rice straw, sugar cane bagasse, *Phanerochaete chrysosporium*

Introduction

Egypt is the largest rice producer in the Middle East region, as cultivated area occupies over 1,080,000 feddan with an average farm yield of 4.76 tons/feddan and an approximate straw production of 2.4 tons/feddan. Both rice and sugar cane cultivation in Egypt, results in an accumulation of large quantities of agricultural wastes (rice straw or bagasse) rich in cellulose, hemicellulose and lignin as illustrated by Mahamud and Gomes, (2012) and Abo State *et al.,* (2014).

Biological and chemical fractionation of RS and Bag are limited due to their complex matrix as well as degrees of lignifications, acetylation and crystallinity in which the polysaccharides, cellulose and hemicellulose are intimately associated with lignin in the plant cell wall **(**Ballerini *et al.*, 1994). Consequently it severely limits the biological hydrolysis of cellulose and other polymers (Chularat and Woranart, 2012).

Therefore, pretreatment is a necessary process for utilization of lignocellulosic material to obtain ultimately high degree of fermentable sugars. Considerable research efforts have been made to improve conversion yields of lignocellulosic materials by the insertion of a pre-treatment step prior to the enzymatic hydrolysis. Both NaClO (1.5%) and dilute NaOH (2%) were effective pre-treatments for lignocellulosic materials with relatively low lignin content of 10 to 18% (Bjerre *et al.,* 1996; El sayed, 2013 and Mohy *et al*, 2015). Fungal cellulases are inducible enzymes that are usually excreted into the environment and depend on cellulose type (amorphous or crystalline) (Ortega *et al.*, 2001).

Munir *et al.,* (2007) found *P. chrysosporium* as the best cellulases producer among other selected fungi species and stated that the time of maximum cellulases activity depends upon the substrate type and fungus. Prachand *et al.,* (2008) revealed that extracellular cellulases secreted by *P. chrysosporium* were capable of hydrolyzing the cellulose into oligo-, tri-, di- and monosaccharides.

The production of cellulases as a key factor in the hydrolysis of cellulosic material must be economically viable among commercial production by *Trichoderma sp., Aspergillus sp.* and *Phanerochaete chrysosporium* (Marcelo *et al.,* 2016).

Therefore, in the present study an attempt had been made for evaluating sequential or successive production of cellulases and saccharification on either RS or Bag, chosen as cheap and

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available wastes substrates, for cellulase production by *P. chrysosporium*, compared to CMC as control.

Materials and Methods

Pretreatment of Rice Straw and Bagasse:

Rice straw collected from the Egyptian local market, was cut, sieved to pass through 3 and 5 mm sieves, pretreated either by soaking in alkaline hypochlorite (1.5% NaClO) at pH 11.5 (RS_1) (wise *et al.,* 1946) and/or in 2% NaOH (RS_2) for 24 hrs at room temperature, (Mohy *et al.,* 2015). Pretreated RS was filtered, neutralized by washing with tap water and oven-dried. Bagasse collected from local sugar cane juice shops was sun dried and hammer milled and used either unwashed and untreated (Bag_1) (Kareem *et al.,* 2016) or treated with 2% NaOH (Bag_2) (Mohy *et al.,* 2015) .

Fungal strain:

P. chrysosporium was generously supplied by Microbiology Department (SWERI-ARC), Giza, Egypt.

Starter medium:

P. chrysosporium was enriched in Mandels medium, of pH 5 (Mandels *et al.,* 1974) on orbital incubator shaker at 38° C (125 rpm) for 5 days.

Cellulases production:

In 500 ml Erlenmeyer flasks, 20 g of pretreated rice straw (RS_1, RS_2), untreated unwashed bagasse (Bag_1), treated (Bag_2) or 10 g CMC (as control) were supplied individually with 160 ml Mandels medium, pH adjusted at 5 and sterilized by autoclaving. The sterilized media in triplicates were inoculated with 40 ml starter medium of *P. chrysosporium*. Flasks were incubated at room temperature for 8 days and examined periodically for cellulases production. Samples filtrates were measured for cellulytic activity.

Sequential and/or successive cellulase production and enzymatic saccharification:

In sequential process (Fig 1-a) ; temperature of the same bioreactors used in cellulase production was raised to 50 °C for 6 and 4 other days, in cases of RS_1 and CMC, respectively. While in the successive process (Fig1-b); the enzyme filtrates were used (as crude enzyme) in saccharifying new RS_1 in new bioreactors at 50 °C at a concentration of 20 g RS_1 / 200 ml buffered enzyme filtrate under aseptic conditions. Samples were daily collected and examined for the released sugars and saccharification % was calculated according to Vallander and Eriksson, (1985).

$$\boldsymbol{saccharification}\ (\%) = \frac{\boldsymbol{reducing\ sugars \times 0.9 \times 100}}{\boldsymbol{total\ carbohydrtate\ of\ RS}}$$

Cellulytic activity assay:

FPase activity was done according to the standard procedure recommended by the commission of Biotechnology IUPAC (Ghose, 1987).

Cellulases activity unit (U):

One U FPase defined as the quantity of enzyme that can release 1µg glucose in 1ml reaction mixture per 1hr under assay conditions.

Estimation of sugars:

Released reducing sugars, in cellulases activity assay, were spectrophotometrically estimated by 3, 5-Dinitrosalicilic acid (DNS) method (Miller, 1959).

Statistical analysis

Data in triplicates were statistically analyzed for the least significant difference (LSD) in completely randomized analysis of variance (ANOVA) at $P \leq 5\%$ calculated using CoStat (6.311) software (Maruthai *et al.,* 2012). Results being designated as small letters arranged in ascending order based on LSD.

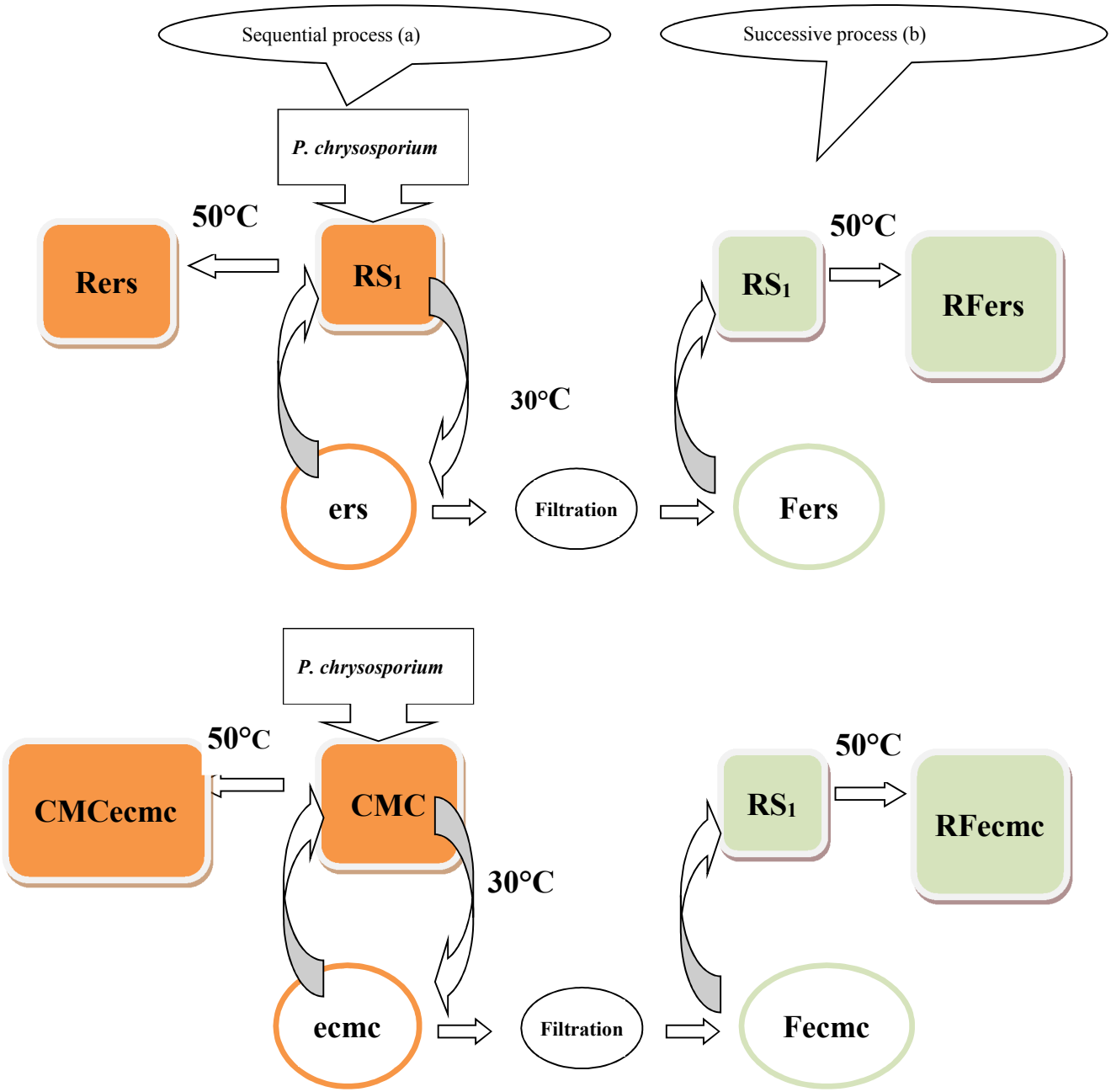

Fig 1. Diagrammatic representation of the sequential (a) production and enzymatic saccharification of RS_1 and/or CMC and successive (b) production and enzymatic saccharification of RS_1

Abbreviation key
Cellulase form type:
ers: non-filtered cellulases produced by *P. chrysosporium* grown on RS
Fers: filtered cellulases produced by *P. chrysosporium* grown on RS
ecmc: non-filtered cellulases produced by *P. chrysosporium* grown on CMC
Fecmc: filtered cellulases produced by *P. chrysosporium* grown on CMC

Saccharified RS form type:
Rers: RS saccharified by non-filtered cellulases produced by *P. chrysosporium* grown on it.
RFers: New RS saccharified by filtered cellulases produced by *P. chrysosporium* grown on old RS
CMCecmc: CMC saccharified by non-filtered cellulases produced by *P. chrysosporium* grown on it
RFecmc: New RS saccharified by filtered cellulases produced by *P. chrysosporium* grown on CMC

Results and Discussion

Production of cellulases on Rice straw and bagasse:

A comparison between cellulases production on RS_1, RS_2, Bag_1 and Bag_2 by *P. chrysosporium* was carried out, as shown in figure (2).

Introducing untreated and unwashed Bag_1 in those comparisons so as not to lose free sugars due to washing and not to affect the high hemicellulose content that is considered more double than that in rice straw, beside its lignin content was less than half that in rice straw as mentioned by El-Tayeb *et al.,* (2012).

Data reveal that various significant differences in cellulases activities through which RS_1 was the best substrate among other examined substrates. The highest FPases obtained on the 5th and the 3rd day was 24.14 U and 20.74 U, respectively, followed by that produced on Bag_1 on the 3rd day (19.59 U). In this regard, the maximum FPase achieved by *Aspergillus niger* reached 30 U (Ong *et al.,* 2012 and Chand *et al.,* 2012). Worthy to notice, that lower Fpases activities were produced on RS_2 and Bag_2 pretreated only with alkali.

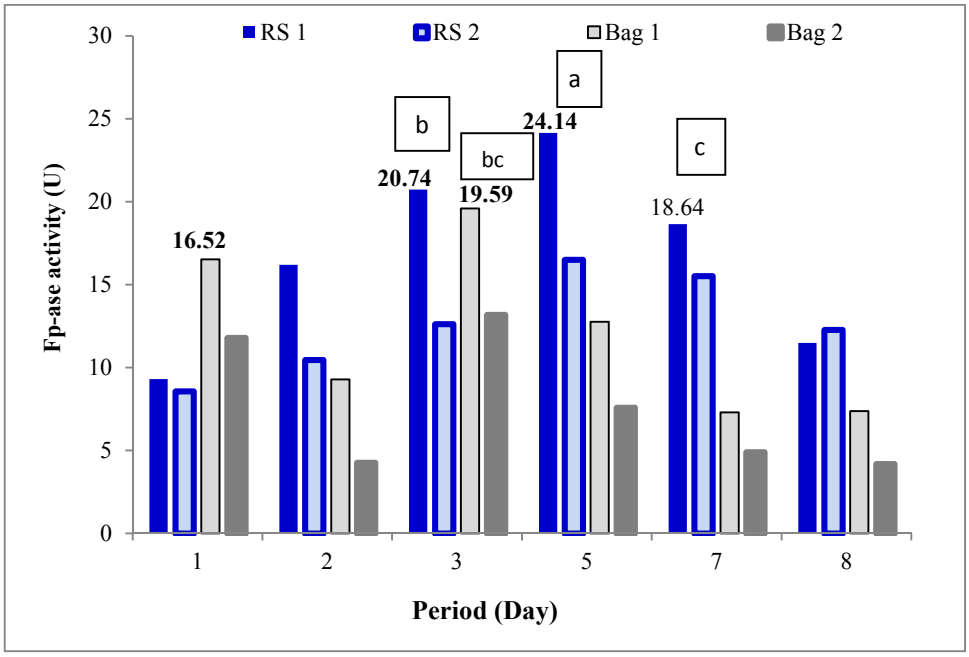

Fig. 2: Production of cellulases by *P. chrysosporium* grown on RS and/or Bag with different pretreatments (LSD 0.05=1.44).

As cellulytic activity within both RS_1 and RS_2 gradually increased up to the 5th day then decreased, it was in agreement with Marcelo *et al.,* (2016) who recorded the highest FPase activity of *P. chrysosporium* after 5 days. On Bag_1 and Bag_2, the cellulytic activity increased on the 1st day then decreased before reaching its maximum on the 3rd day. Induced cellulases production might be due to the presence of amorphous part of the substrate with less crystallinity as a result to previous chemical treatment. It was remarkable that Bag_1 showed higher FPase activity than Bag_2 on the 1st day. It may be due to that the former resulting from sugar extraction process contains residual free sugars that might play a role in the first 24 hr of incubation in increasing fungal load leading to increase in cellulases producing force. Moreover, hemicellulose content in Bag_1 may be higher than Bag_2 making it more available for cellulytic induction and action. This was in agreement with Mohy *et al.* (2015) who stated that Egyptian untreated and treated bagasse with alkali had hemicelluloses content of 28% and 10%, respectively.

While the cellulases act on those structures, more reducing sugars were released leading to their accumulation and back inhibition causing decrease in observed activity. Mostly the fungi continued to produce additional cellulases through progressing time, after which complete blocking

action by the remaining crystalline part of substrate and possible depletion of needed nitrogen source caused decline in cellulases production and consequently lower FPases final activity.

Munir *et al.,* (2007) attributed the decrease in cellulase activity to cellobiose released during cellulytic action which is known as cellulases inhibitor which is in agreement with our results demonstrating a decline in cellulase production after 5 days.

Gupta *et al.,* (2009) clarified that cellulases first attack the easily accessible amorphous regions of the substrate which was translated to the higher initial rate of hydrolysis. Furthermore, it was difficult to hydrolyze crystalline regions of cellulose giving resistance to the enzymatic hydrolysis. Chularat and Woranart (2012) stated that the most enzymatic solubilization rates were reversibly correlated to percentages of crystallinity.

Comparison between *P. chrysosporium* cellulases produced on RS_1 and/or CMC:

As RS_1 was the best substrate for cellulase production with higher Fpase activity, it was selected for further studies in comparison with CMC as the sole carbon source to induce *P. chrysosporium* cellulase production, as shown in figure (3).

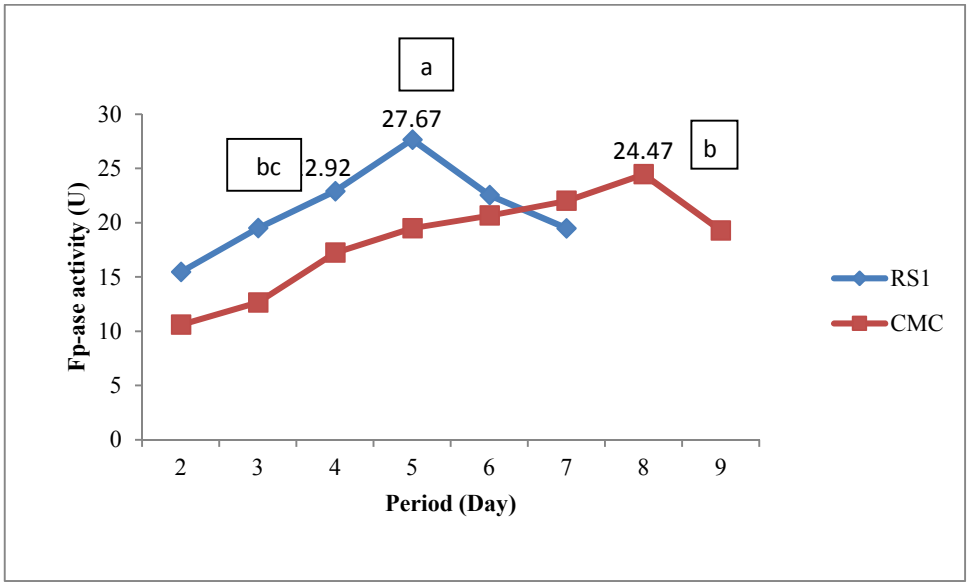

Fig 3. *P. chrysosporium* cellulases produced on RS_1 and CMC (LSD 0.05=2.12)

Results referred to a significant difference between activities of cellulases produced on the RS_1 and that on the CMC. Remarkable difference as well was observed for the maximum activity peak duration, as the highest activity was monitored by the 5th day (27.67 U) for RS_1 and by the 8th day (24.47 U) for CMC. These results are in agreement with Rajoka (2004) who clarified that the fungus synthesizes enzyme with lower activity when grown on soluble carbon sources like CMC while it was higher when grown on cellulosic substrates.

Worthy mention, that CMC is a highly specific substrate inducing endo-acting cellulases production. The endo cellulases create amorphous sites that are ideal for further degradation by other cellulases types. This revealed that CMC when was used as a substrate for *P. chrysosporium* growth and cellulase production, only endo cellulases were induced. This lead to slow substrate utilization that in turn had no back inhibition (catabolic repression) as noted by Munir *et al*., (2007).

Sequential cellulase production and enzymatic saccharification:

The processes were carried out on RS_1 by *P. chrysosporium* compared with CMC as the sole carbon source by raising the temperature in the same individual bioreactor used in cellulase production to 50°C after reaching maximum cellulytic activity for calculating the saccharification % of the residual RS_1 and/or CMC.

Around 26.29 U and 23.83 U of cellulases were produced by *P. chrysosporium* by the 5th and the 8th day when grown on RS_1 and CMC, respectively. About 50.4% from RS_1 was saccharified by the 10th day (5th day from the beginning of saccharification) as shown in figure (4). On the other hand, 39.12% from CMC was saccharified by the 11th day (the 3rd day from the beginning of saccharification) as shown in figure (5). The decrease in the saccharification % after that may be due to accumulation of glucose and cellobiose which might suppress the cellulase activity (Prachand *et al.,* 2008) and that the fungus may consume the excess of sugar released at the end of saccharification. Chand *et al.,* (2012) mentioned that, in sequential cellulase production and saccharification, that 76% of saccharification of alkali pretreated RS was achieved and that was due to more enzymes involved in hydrolysis.

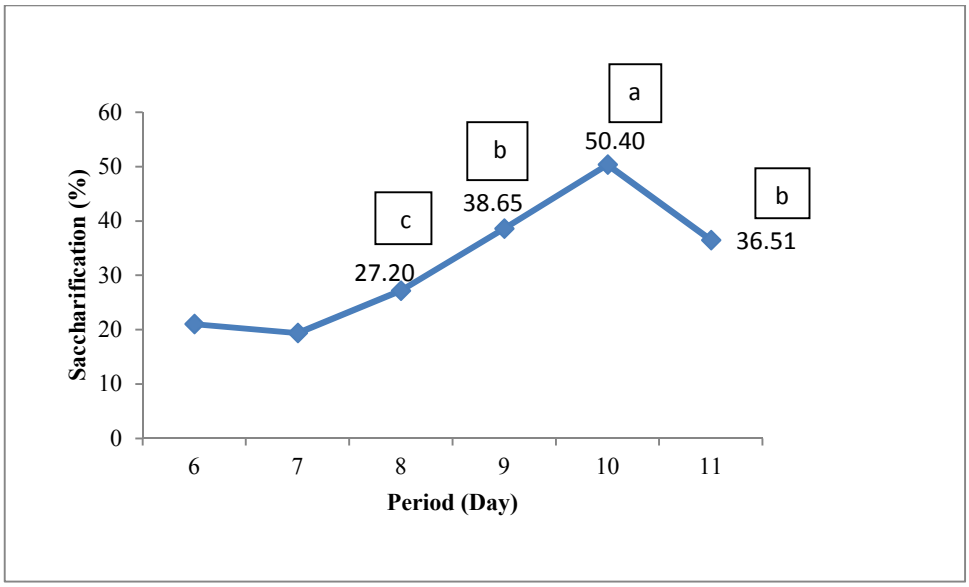

Fig 4. Saccharification of RS_1 using *P. chrysosporium* cellulases (LSD 0.05=4.26)

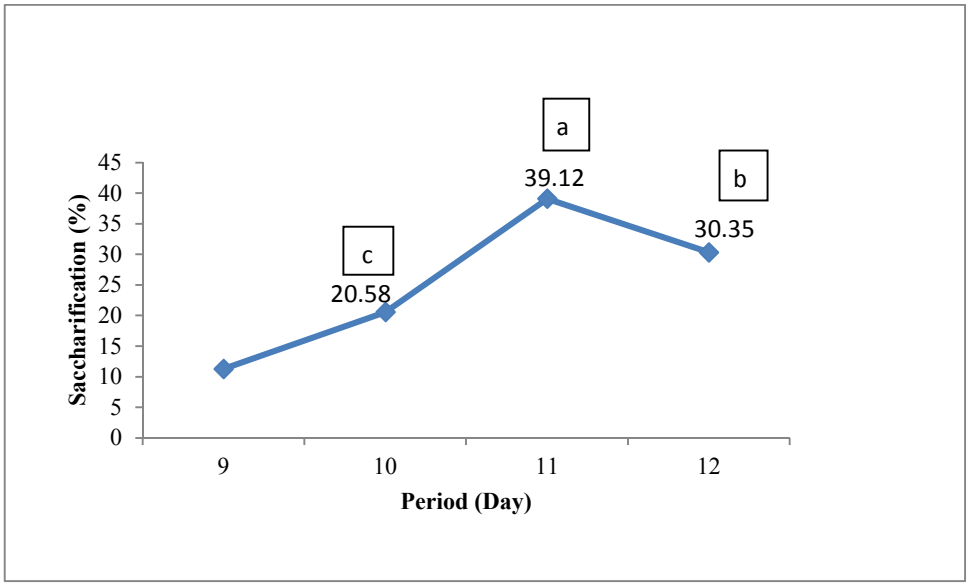

Fig 5: Saccharification of CMC using *P. chrysosporium* cellulases (LSD 0.05=1.88)

Utilization of non-filtered and filtered cellulases in RS_1 and/or CMC saccharification:

A comparison was done between a sequential and a successive process for cellulases production and saccharification. In case of sequential process, the saccharification of the same substrates (Rers and CMCecmc) was done in the same bioreactors using the same enzyme produced on it without filtration (ers and ecmc). While in case of successive process, filtered crude enzymes (Fers and Fecmc) produced by *P. chrysosporium* grown on either RS_1 or CMC, having activities approximately of 30 U and 27 U, respectively, were used in saccharifying new RS_1 (RFers and RFecmc) in new bioreactors for measuring released sugars and its saccharification percentage.

It can be concluded from figure (6) that there was a significant difference between the same (Rers) saccharified by (ers) and all the other substrates. About 54.24% of (Rers) was saccharified by the 5th day which was higher than new (RFers) which was saccharified by (Fers) (49.7% on both 5th and 6th day) followed by (CMCecmc) with 41.9% saccharification by (ecmc) by the 3rd day.

This difference might be due to the presence of living fungal cells which acted on the same Rers releasing more glucose. Saccharification of both Rers and CMCecmc decreased after the 5th and 3rd day, respectively. It is known that wood-decaying fungi are generally mesophilic with an optimum temperature for growth between 20 -30 $^{\circ}$C. However, *P. chrysosporium* which can grow in hot environments such as self-heating piles of wood chips has an optimum temperature for growth of 40 $^{\circ}$C and continues to grow at up to 50 $^{\circ}$C (William, 2015). Based on this fact, living fungal cells in the crude enzyme (ers or ecmc) might have grown during saccharification process (at 50 $^{\circ}$C) consuming the excess of sugar released at the end of saccharification. On the other hand, saccharification of new RFers and RFecmc by (Fers and Fecmc) remained at stable level, meaning that introduced cellulases reached its maximum capacity.

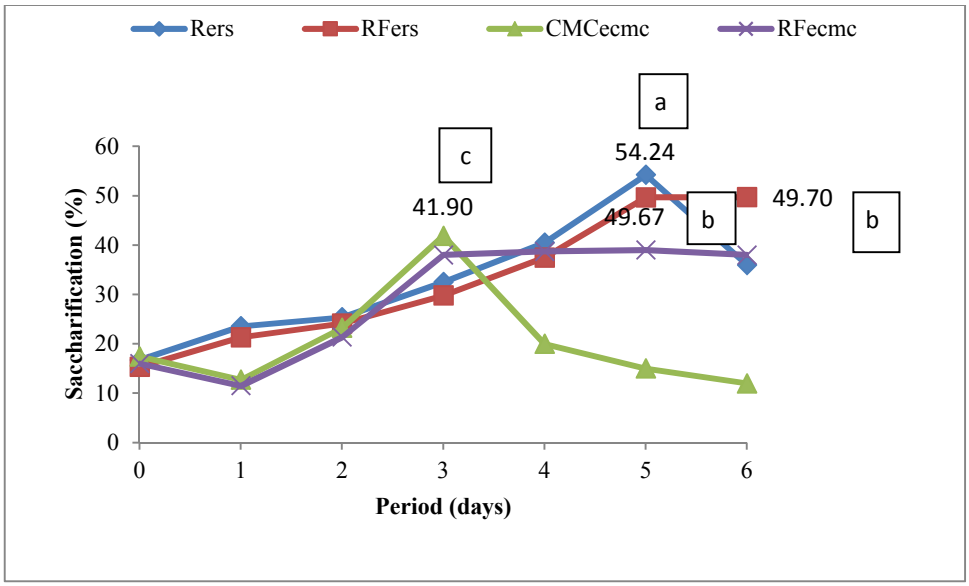

Fig 6: Saccharification of same RS_1 and/or CMC by non-filtered (ers and ecmc) and new RS_1 by filtered *P. chrysosporium* cellulases (Fers and Fecmc) (LSD 0.05=0.95)

Conclusion

Pretreated rice straw with sodium hypochlorite was more efficient than other substrates examined for cellulases production by *P. chrysosporium*. Utilization of non-filtered *P. chrysosporium* cellulases in sequential process for cellulases production and saccharification was more economic than the successive process. Therefore, production of ethanol from saccharified RS will be examined in the further study.

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