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PRODUCTION OF CELLULASE AND XYLANASE FROM *CANDIDA TROPICALIS* (MK-118) ON PURIFIED AND CRUDE SUBSTRATES

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Abstract

Lignocellulose, a mixture of cellulose, hemicellulose and lignin, is naturally occurring chemical feedstock on earth which can serve as substrate for the production of plant cell wall degrading enzymes (PCWDE) including cellulase and xylanase. Yeast strains have scarcely been reported for the simultaneous production of more than one PCWDEs. In this study, an indigenous strain of *Candida tropicalis* MK-118 has been reported for the potential to co-produce endoglucanase (EG), β-glucosidase (BGL) and xylanase (Xyl). It was observed that the production of an individual enzyme varied differently with a change in cultivation conditions. For EG and BGL production, cultivation at 40°C was more suitable while the highest titers of Xyl were obtained at 25°C. Higher yields of EG and Xyl were obtained under neutral pH, whereas, acidic medium favored BGL production. Although, an inoculum size of 2% was found appropriate for all the three enzymes, however, maximum production of EG, BGL and Xyl was obtained in presence of 0.5% carboxymethyl cellulose, 1.5% salicin and 1% xylan. Furthermore, the enzymes were characterized for their optimal activities. The strain showed ability to ferment sugarcane-baggase and wheat-bran with the concomitant production of cellulases and xylanases. Interestingly, the strain also produced alcohol in complex medium. *C. tropicalis* MK-118 may find its application for the production of industrially important enzymes and alcohol under submerged fermentation of crude substrates. The properties of enzyme preparation were industrially relevant and hence the enzyme can also be applied for various processes.

Key words: *Candida tropicalis*, Endoglucanase, β-glucosidase, Xylanase, Ethanol, Sugarcane bagasse.

Introduction

Yeasts are known for their biotechnological applications for many centuries. They are found in many habitats along with filamentous fungi and bacteria, though, less abundantly (Chanchaichaovivat *et al.*, 2007). The ability of yeasts to produce ethanol has not been replaced by any other group of microorganism till date. However, the production of plant cell wall degrading enzymes (PCWDE) is not common among yeasts, therefore, they cannot be applied directly to convert lignocellulosic (LC) biomass to ethanol and hence are either co-cultured with bacteria or molds, or are engineered with the required genes.

LC masses are rich in fermentable carbohydrates i.e. cellulose and hemicelluloses and are considered as cost effective raw materials for fermentation industries. Attention towards LC materials is ever increasing considering the abundant quantities available as domestic, agro-industrial and forestry wastes (Howard *et al.*, 2003). However, the effective utilization of such wastes requires pretreatment procedures, either through physico-chemical or biological methods. Biological methods are mainly based on application of microbial enzymes, cellulase and xylanases (Xyl).

Cellulases catalyze degradation of the most abundant biopolymer, cellulose. Various component enzymes, including endoglucanase (EG), exoglucanase (EXG) and β-glucosidase (BGL) act synergistically (Yang *et al.*, 2003) to completely degrade cellulosic substrates (Lynd *et al.*, 2002).

Xylan in the most abundant hemicellulose which is degraded by xylanase (Xyl) that hydrolyzes β-1,4-glycosidic bond present in xylan structure and convert it into xylo-oligosaccharides (Polizeli *et al.*, 2005). Together with cellulases, Xyl find various applications in commercial and industrial processes, notably in improving the nutritional quality of silage, clarification of juices and wines, paper and pulp industry and de-inking processes (Kulkarni *et al.*, 1999).

The role of cellulase and Xyl in bioconversion of LC masses necessitates their large scale production, for which fungal or bacterial strains, are employed. The strains of *Trichoderma reesei* (Lynd *et al*., 2002), *Aspergillus* (Sohail *et al*., 2014, 2015) and *Bacillus* (Tariq *et al*., 2018) are employed for the industrial production of cellulase or Xyl. Although, very few of the native yeast strains have been studied in this regard (Olofsson *et al.*, 2008), however, recent reports on the production endoglucanase from *S. cerevisiae* MK-157 (Shariq *et al*., 2018) and Xyl production from *C. tropicalis* MK-160 (Shariq & Sohail, 2018) indicated the potential of indigenous yeast mycoflora for possible application in waste biorefineries. The present study describes co-production of cellulase and Xyl from an indigenous strain of *Candida tropicalis* and characterization of the enzymes.

Materials and Methods

Organism, screening and inoculum preparation: The yeast strain, MK-118, was isolated from enriched soil and identified on cultural, morphological and biochemical test as described in Kurtzman & Fell (1998). The strain was screened for the extracellular cell-wall degrading enzyme *i.e.* cellulase and Xyl. Initially, it was screened qualitatively using plate method and then by quantitative assays as described by Sohail *et al*., (2014) and Kumari *et al*., (2017). To prepare inoculum, a single colony from Sabouraud dextrose agar (SDA) plate was transferred to SDB and incubated it at 40°C until optical density (OD) at 600 reached to 1.0. From this tube, an aliquot was transferred to the production medium to achieve 5% final concentration.

Factors affecting cellulase and xylanase production and activity of the enzymes: The multiple factors affecting cellulase and Xyl production by the strain MK-118 were optimized by adopting traditional strategy to vary one factor at a time and keeping all the other factors constant. The effect of production media was studied by transferring inoculum to three different media, mineral salt medium (MSM, Shariq & Sohail, 2018), Yeast extract-peptone medium (YEPS) and Peptone medium (PM), containing carboxymethyl cellulose (CMC) for EG, salicin for BGL or beechwood xylan for Xyl. Flasks (in triplicate) were incubated at 40°C for 72 h at 150 rpm. Post incubation cell-free culture supernatant (CFCS) was obtained by centrifugation at 4500 xg and assayed for EG, BGL (Sohail *et al.*, 2015) and Xyl activities (Kumari *et al.*, 2017) using dinitrosalicylic acid method (Miller, 1959). The optimum production temperature for EG, BGL and Xyl was determined by cultivating MK-118 in the corresponding suitable medium at different temperatures from 25° to 40°C followed by assaying CFCS. Likewise, to determine optimum pH for the production of the three enzymes, MK-118 was cultivated in appropriate medium with pH adjusted to 4.0, 5.0, 6.0 or 7.0 at the corresponding optimum temperature. Similarly, the studies on other parameters were carried out by varying carbon and nitrogen source in the medium, concentration of the substrate or inducer of the enzyme and inoculum size. Finally, the growth and enzyme production kinetics was studied by cultivating the strain under optimum conditions in the suitable medium for 72 h, aliquots were taken intermittently and OD_{600} was noted. CFCS obtained from the aliquots were assayed for the EG, BGL and Xyl activity.

The enzyme preparation was studied to optimize the conditions (including temperature, pH and substrate concentration) for the activities (Shariq & Sohail, 2018). The effect of metallic ions was also investigated as described by Sohail *et al*., (2014).

Fermentation of sugarcane-bagasse and wheat-bran: The ability of the strain to utilize two of the crude substrates, Sugarcane-bagasse (SB) and Wheat-bran (WB), for the co-production of cellulase and Xyl under submerged fermentation was evaluated. SB and WB were collected from a local market, sun dried, oven dried at 50°C for 24 h and ground to 100 mesh size. Two separate pretreatments to SB and WB were employed using alkali and hydrogen peroxide (H_2O_2). Briefly, 1% KOH (in solid liquid ratio 1:4) and 2% solution of hydrogen peroxide (0.5 gm H_2O_2/gm biomass) were separately loaded on the powdered substrates for 1 h at room temperature. After pretreatment, SB and WB were washed excessively with distilled water to remove residual chemicals and were dried until constant mass. For fermentation, 1g of the pretreated SB and WB were added in separate flasks containing 100 mL MSM and sterilized by autoclaving. After inoculating with the strain MK-118 the flasks were incubated under previously known optimized conditions for 4 days. Post-incubation supernatants were collected by centrifugation and were assayed for the EG, BGL and Xyl activities (Miller, 1959).

Alcohol production and dye-absorption: Two possible biotechnological application of the strain MK-118 were investigated i.e. to produce alcohol and to adsorb azo-dye. Initially, MK-118 was cultivated in SDB containing varying amount of alcohol ranging from 0% to 16% for 72 h at 30°C and growth was monitored by measuring absorbance spectrophotometrically at 600 nm. Consequent to the findings that the strain can tolerate alcohol, the strain was inoculated in SDB and alcohol production was monitored according to Shukla *et al*., (2011).

Dye-adsorption ability of MK-118 was evaluated by making various concentration of a test azo-dye, congo red, from 20-100 ppm. MK-118 was grown in 10 mL of SDB for 48 h until OD_{600} reached to 1.0, cells were harvested by centrifugation at 4500 xg and were transferred to congo red solution. Dye adsorption was investigated by taking OD_{497} of the solution intermittently.

Results and Discussion

Bacteria and fungi remain organisms of choice for the large scale production of cellulase and Xyl. Yeasts have less frequently been reported, particularly for the co-production of the two enzymes. Although, production of a single enzyme facilitates downstream processing, however, multienzyme preparations are of particular interest for the saccharification of crude substrates and the producer may find their application in waste biorefinery processes. In this context, out of more than hundred yeast strains, the strain MK-118 was selected for the production of EG, BGL and Xyl.

Identification of the strain and screening for cellulase and xylanase production: The strain, MK-118, retrieved from the departmental collection, was originally isolated from soil sample using enrichment technique. It was identified on the basis of cultural, morphological and biochemical tests. The colonies were round, cream in color, entire margin with raised elevation and budding was observed. It was able to ferment only glucose, maltose and sucrose. It did not show growth in presence of 1% acetic acid and was found negative for urease production and citrate utilization and positive for nitrate reduction. It showed characteristic metallic blue color colonies when streaked on CHROM agar. Based on these results, the strain MK-118 was identified as *Candida tropicalis*. Although, the strains of *C. tropicalis* have been studied for their significance in infectious diseases, however, few of the strains have shown to have biotechnological applications as discussed by Kumar *et al*., (2018) where *C. tropicalis* MTCC 6192 was studied to utilize corncob hydrolyzate for the production of xylitol.

The strain MK-118 was screened qualitatively by cultivating for 48 h at 30°C on MSM-agar plates containing CMC, salicin or xylan as a substrate for EG, BGL and Xyl, respectively. Plates were analyzed for the zones around the colonies. The results inferred that MK-118 had the ability to produce all the three enzymes. After the qualitative analysis, MK-118 was quantitatively screened by cultivating in MSM broth supplemented with corresponding substrates of the enzymes. The strain produced 7.44, 3.06 and 2.8 IU mL^{-1} of Xyl, EG and BGL, respectively.

Earlier, strains of *C. tropicalis* have been reported for Xyl (Shariq & Sohail, 2018; Nouri *et al.,* 2017) and cellulase (Sulman & Rehman, 2013) production. Preparations comprising of both the enzymes, cellulase and xylanase, are essentially required for the degradation of complex and recalcitrant LC substrates. However, simultaneous production of cellulase and xylanase, as reported by Thongekkaew *et al.,* (2014) from *C. easanensis*, is not common among yeasts and hence provided merit to study *C. tropicalis* MK-118.

Optimization of production of EG, BGL and Xyl from MK-118 and their characterization: Microbial enzyme production is influenced by a multitude of nutritional and environmental factors, hence, these are routinely studied to obtain optimum levels of the enzyme in cost effective manner. When the effect of various factors on the production of EG, BGL and Xyl from the strain MK-118 was investigated, it was found that the highest titers of Xyl (10.44 IU mL^{-1}) and EG (7.532 IU mL^{-1}) were obtained in YEP while PEP found to be an appropriate medium for BGL (5.72 IU mL^{-1}) production. The effect of different carbon and nitrogen sources on the production of the enzymes was studied by replacing the sources from the optimized media. The data indicated about the induction of the EG in presence of CMC, whereas, salicin and xylan appeared as inducers for the BGL and Xyl production by the strain MK-118, respectively (data not shown). These results were in agreement with Abou-Taleb *et al*., (2009) who found CMC and yeast extract as optimized sources for cellulase production. Similarly, more titers of Xyl were produced by *Pichia stipitis* in presence of xylan in comparison to simple carbon sources, such as, glucose or xylose (Phongdara & Tumsuwan, 1996). Furthermore, it was observed that 0.5% xylan gave higher yields of Xyl, while 1.5% CMC and 1% of salicin produced the highest levels of EG and BGL, respectively.

Among nitrogen sources tested, urea was found appropriate for the Xyl production, while, the strain produced highest titers of EG and BGL in yeast containing extract medium (Table 1). Generally, it is accepted that organic compounds release less nitrogen than inorganic nitrogenous compounds; however, presence of other constituents in organic compounds favors the growth and hence supports the enzyme production.

Temperature is considered as one of the most important environmental factors that influences the enzyme yield from a microorganism. *C. tropicalis* MK-118 produced the highest titers of Xyl and EG at 40^{o}C, while for the production of BGL, 25°C was the most suitable cultivation temperature. An optimum temperature for the production of BGL was different than for EG and Xyl, along with few other factors, indicated different regulation of this enzyme than the other two enzymes. Previously, a temperature of 40°C was reportedly found appropriate by Ray *et al*., (2007) for the cellulase production from *B. subtilis* and *B. circulans.* In contrast to this finding, the best temperature for the production of Xyl from *A. awamori* was reported as 30°C by Smith & Wood (1991). Nevertheless, the production of enzyme at higher temperature is advantageous for operating a process in tropical countries to minimize the cost on maintaining the temperature.

An increment in the initial pH of the production media resulted in the increase in the productivity of Xyl and EG by *C. tropicalis* MK-118 until it attained a peak at pH 7.0.While acidic medium (pH 5.0) favored the production of BGL (Table 1). The finding was in agreement with previous studies where pH 7 was reported as optimum pH for EG (Abou-Taleb *et al.,* 2009) and 6.5 for Xyl production (Liu *et al.,* 1998).

Inoculum size has a remarkable effect on the enzyme production as inoculating fewer cells incurs delay to attain an appropriate cell density. On the other hand, a higher inoculum may hinder the process by interfering with effective mass transfer. A lower inoculum size (2%) was found appropriate for the production of all the three studied enzymes (data not shown) compared to the inoculum size (3%) reported by Ray *et al*., (2007).

The strain MK-118 of *C. tropicalis* was cultivated under optimum conditions and the kinetics of growth and EG, BGL and Xyl production were studied, separately. The results revealved that although the strain MK-118 remained in the log phase of its growth for 72 h in the xylan containing medium, however, the Xyl titers attained their peak after 52 h with a concomitant increase in the amount of reducing sugars (RS) in the medium indicating active utilization of the substrate by the strain. Whereas, pH of the medium did not vary to a great extent during cultivation (Fig. 1a). In CMC containing medium, MK-118 grew exponentially for 76 h with an increasing trends of the EG production and release of RS (Fig. 1b). A similar trend was observed when the strain MK-118 was cultivated in salicin containig medium where duration of the exponential phase of growth and BGL production was 72 h. pH of the medium, however, decreased to 5 at the end of the experiment in this case (Fig. 1c).

When EG, BGL and Xyl from MK-118 were characterized for the optimum temperature and pH, results inferred that the maximum activity of Xyl was observed at 40°C which was in agreement with Phongdara & Tumsuwan (1996) while optimum temperature for EG and BGL activity was found as 30°C (Table 2). All the three enzymes showed sustained activity under a wide range of pH (data not shown) hence slightly higher activities attained at specific pH that were referred as optimum pH.

Table 1. Optimized parameters for the production of the enzymes from *Candida tropicalis* MK-118.

Parametrs	Xylanase	Endoglucanase	β-glucosidase
Production media	YEP	YEP	PEP
Temperature	40 ˚C	40 ˚C `	25 ˚C
pH	7 pH	7 pH	5 pH
Carbon source	Xylan	CMC	Salicin
Nitrogen source	Urea	Yeast extract	Yeast extract
Substrate concentration	0.5 % (Xylan)	1.5 % (CMC)	1 % (salicin)
Inoculum size	2 %	2%	2 %

Table 2. Optimized parameters for the activity of the enzymes from *Candida tropicalis* MK-118.

Parametrs	Xylanase	Endoglucanase	β-glucosidase
Temperature	40˚C	30˚C `	30˚C
pH	5.5 pH	3.5 pH and 10.5 pH	8 pH
Substrate concentration	1.5 % (Xylan)	2 % (CMC)	1.5 % (salicin)

Table 3. Effect of metallic ions and chemicals on enzymatic activities by *Candida tropicalis* MK-118.

Chemical	Enzyme activity (%) in presence of chemicals at concentration					
	Xylanase		Endoglucanase		B-glucosidase	
	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM
Control	100	100	100	100	100	100
NaCl	91%	93%	79.8	77.65	87.1	90.1
$MgSO_4.7H_2O$	98.7	104.26	100	101.1	63.31	75.1
KH_2PO_4	108.43	94.9	88.65	87.9	86	78.9
$CaCl_2$	106.34	110.5	75.2	68.1	93.1	81.8
$MnSO_4.5H_2O$	78.54	97.9	78.7	51.1	81.8	39.8
KCl	86.5	96.6	63.82	77.97	47.8	30.7
$CoCl_2$	109.8	88.28	3.5	2.5	81.1	78.1
$CuCl_2$	85.49	84.8	67.37	66.6	84.1	86
UREA	108.43	109.46	2.41	1.78	69.1	90.1
EDTA	79.9	91	67.8	69.4	75.1	75.1

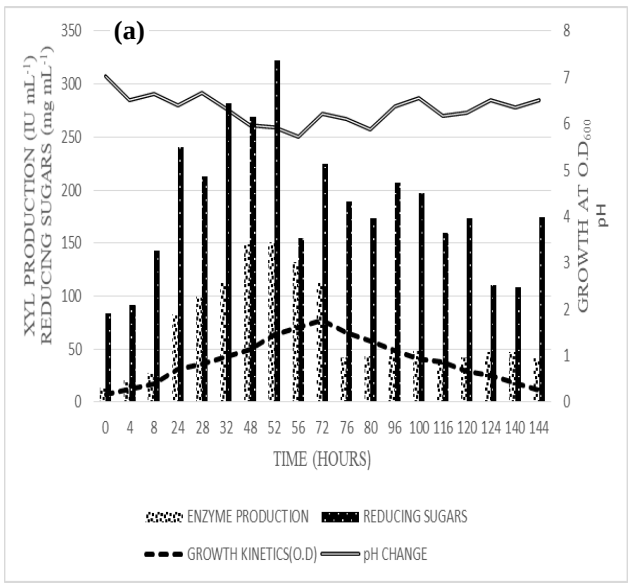
In addition to optimizing parameters influencing the enzyme production, the factors affecting enzyme activities were also investigated. The studies on the effect of the substrate concentration on activities of the enzymes manifested no significant difference in the Xyl activity with varying substrate concentration, however, slightly higher activity was observed with 1.5% of xylan. Maximum EG and BGL activities were observed with 2% CMC and 1.5% salicin, respectively (Table 2). In contrast, Baharuddin *et al*., (2016) reported a lower substrate requirement (1% of CMC) for optimum cellulase activity. The difference in substrate requirement can be linked with the affinity of the enzymes towards their substrates.

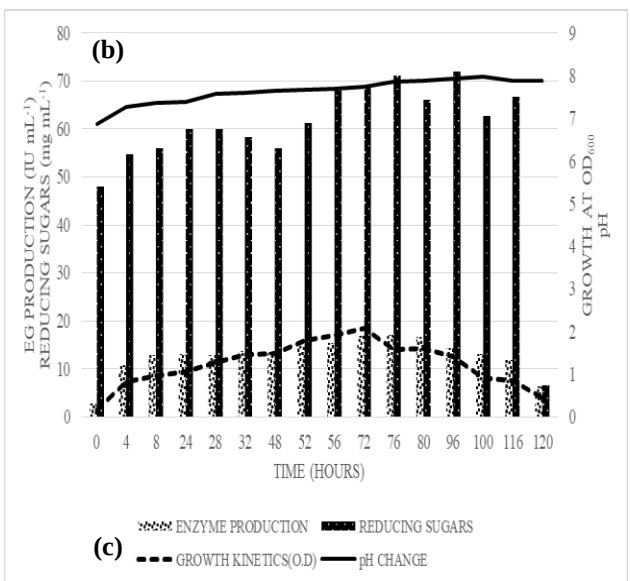
Since, LC substrates frequently get contaminated with chemicals, therefore, it is imperative to study the effect of different chemicals on cellulolytic and xylanolytic preparations. These chemicals can either activate the enzyme by stabilizing the structure or can bind to the amino acid of the enzyme or to substrate thereby altering the accessibility of the substrate and inhibit the enzyme activity. In this study, the effect of chemicals was evaluated at two different concentrations i.e. 1 and 5 mM. An enhancement in the Xyl activity in presence of $MgSO_4.7H_2O$, KH_2PO_4, $CaCl_2$, $CoCl_2$ and urea was observed at either concentration, while rest of the ions and EDTA inhibited this activity (Table 3). On the other hand, urea and $CoCl_2$ found to be inhibitor for the EG activity. Likewise, KCl, $MnSO_4$, $MgSO_4$ and $CuCl_2$ inhibited the BGL activity (Table 3). Previously, Liu *et al*., (1998) reported the significant inhibitory effects of Mn^{+2}, Hg^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+}on the Xyl from *Trichosporon cutaneum* SL409.

After the optimization of parameters affecting the enzyme production and activity, Xyl, EG and BGL levels reached to 21.292, 17.39 and 12.32 IU mL^{-1}, respectively.

Fermentation of sugarcane-bagasse and wheat-brane: Being an agricultural country, Pakistan is a house of multitude of agro-waste materials that are usually under-utilized. The utilization of such LC masses as raw materials offers prospects to establish fermentation industries in the country. SB and WB are among such LC wastes that are generated in huge quantities. Padilha *et al*., (2015) obtained 0.381 IU mL^{-1} of EG using SB as substrate. Similarly, WB was found suitable for the production of Xyl by *Tricoderma orientalis* (Long *et al.,* 2017). In the present study, these two substrates were taken as the main carbon source in fermentation media and the production of EG, BGL and Xyl by *C. tropicalis* MK-118 was investigated. The results infered higher production of Xyl when alkali pretreated SB or WB was fermented than untreated or H_2O_2 treated substrates (Fig. 2). While pretreatment of the substrates did not affect fermentative production of EG to a greater extent. Nonetheless, the results showed that the strain MK-118 could be used to produce one of the three enzymes (EG, BGL and Xyl) by cultivating it in presence of commercially purified substrate or it could also be employed for the production of a multienzyme by cultivating on crude substrate such as SB, hence, it can fulfill the requirements of diversified industrial processes.

Ethanol production and dye adsorption: In addition to the produciton of the enzymes, the strain MK-118 was evaluated for its other roles in biotechnology sector. Knowing that the strain exhibited good growth (<1 OD_{600}) in presence of alcohol, the strain was cultivated in complex medium containing 5% glucose and production of ethanol was monitored. A yield of 5 % ethanol was obtained that was comparable to the reports by Gupta *et al*., (2009) using *Pichia stipitis* and *S. cerevisiae*; while it was lower than obtained from *C. tropicalis* grown on olive pruning (Martin *et al.,* 2010). Nonetheless, the ability of the strain to ferment crude substrates with the concomitant production of cellulases and xylanases, along with the production of ethanol on complex medium necessitates further studies to assess possible candidature of this strain for biorefinery purposes.





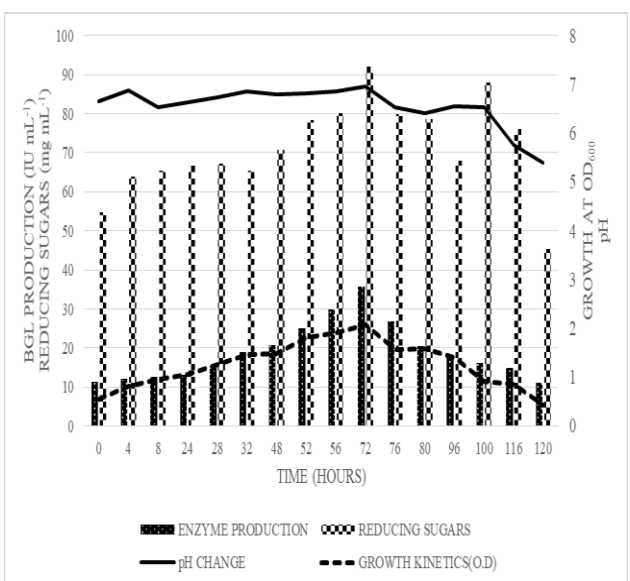


Fig. 1. Growth and (a) Xyl (b) EG (c) BGL production kinetics from *Candida tropicalis* MK-118 on commercially purified substrates.

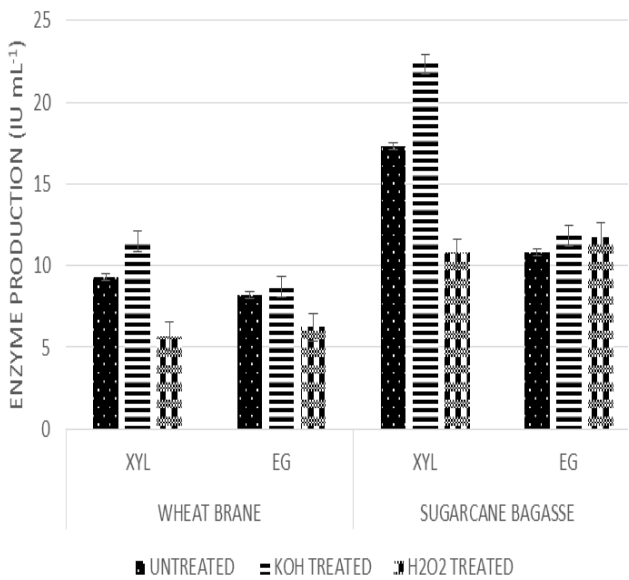


Fig. 2. Endoglucanase (EG) and Xylanase (Xyl) production from *Candida tropicalis* MK-118 under submerged fermentation using untreated and pretreated sugarcane bagasse (SB) and wheat bran (WB).

Table 4. Adsorption of Congo red by the strain MK-118.

Congo red (ppm)	Bioadsorption (%) in time duration (h)			
	2	4	24	29
20	57.903	61.34	58.2	89.347
30	54.469	59.078	67.877	90.22
40	66.502	69.629	78.024	94.73
50	53.098	55.137	65.803	95.4509
60	64.026	65.805	73.8	95.325
70	46.396	48.152	90.066	96.244
80	63.5	59.16	70.944	95.229
90	53.54	54.779	60.9218	95.7409
100	63.699	64.065	70.813	94.065

Yeasts cells have been reported for their ability to absorb dyes and hence are considered to be applied for bioremediation of textile waste which usually contains azo-dyes. In Pakistan, textile sector contributes in the generation of huge revenues but at the same time it also releases toxic waste. A test azo-dye, congo red, was included in this study to observe the adsorption of this dye using cells of *C. tropicalis* MK-118. The experiments showed that the strain had ability to decolorize 100 ppm solution of congo red to 94% (Table 4) that was higher than that of obtained by Ozfer *et al*., (2003) where fungal pellets could remove more than 75 % of the dye color in 24 h. The adsorption or decolorization of the dye might have resulted in the formation of colorless aromatic compounds (Tan *et al*., 2013) that need to be investigated in detail before speculating the role of MK-118 to remediate the dye-ridden environment.

Conclusion

C. tropicalis MK-118 can produce cellulase and xylanase as homogenous preparation when cultivated on commercially available substrates, whereas the strain can produce multienzyme when cultivated on crude substrates. The production of all the three enzymes is

growth related and influenced greatly by a change in environmental temperature. The strain can also produce significant titers of alcohol on complex medium and the cells can be used to adsorb an azo-dye, congo red.

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