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Hyper production of carboxy methyl cellulase by *Thermomyces dupontii* utilizing physical and chemical mutagenesis

Hiperproducción de carboximetilcelulasa por *Thermomyces dupontii* utilizando mutagénesis física y química

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Abstract

Utilization of cellulases as substitute of chemical process gained huge momentum in the field of biotechnology. Now there is dire need to find out un explore reveres of fungi possessing greater potential for efficient cellulase production. This boosted isolation of novel thermo tolerant fungal strains capable of producing the targeted product. In this investigation 70 thermophilic cellulolytic fungal strains were isolated. All the strains were screened via submerged fermentation. The strain showing highest CMCase activity was identified by conventional method i.e. based on morphology and microscopic features and confirmed by 18S rDNA gene sequencing, using specific ITS primers. The modified CTAB method was used for rapid extraction of DNA from thermo tolerant strain. The selected strain subsequently subjected to sequencing and phylogenetic analysis. The result indicates the selected strain was found to be *T. dupontii*. For strain improvement the *T. dupontii* was subjected to random mutagenesis by using physical mutagen i.e. (UV) irradiation and chemical mutagen i.e. EMS treatment. Out of 40 screened mutant *T. dupontii* EMS 15 had 2.4 fold more yield (21.8 U/ml/min) as compared to parental wild strain. The Five fermentation media were also screened. The Medium3 gave higher titer of cellulase activity (29.3 U/ml/min) and found to be the best medium.

Keywords: molecular identification, fungi, CMCase, mutation.

Resumen

El uso de celulasas ha Ganado un gran impulse en el campo de la biotecnología como sustituto de procesos químicos. Existe la necesidad de explorar hongos con potencial capaces de producir celulasa. Este aislamiento potenciado de nuevas cepas fúngicas termotolerantes capaces de producir el producto objetivo. En esta investigación se aislaron 70 cepas de hongos termofílicos celulolíticos. Todas las cepas fueron analizadas a través de la fermentación sumergida. La cepa que muestra la mayor actividad de CMCase se identificó mediante el método convencional, es decir. basado en la morfología y las características microscópicas y confirmado por la secuenciación del gen 18S rDNA, utilizando iniciadores específicos ITS. El método CTAB modificado se utilizó para la extracción rápida de ADN de cepa termotolerante. Posteriormente, la cepa seleccionada se sometía a análisis de secuenciación y filogenético. El resultado indica que la cepa seleccionada fue y se encontró que era *T. dupontii*. Para mejorar la cepa, el *T. dupontii* fue sometido a mutagénesis aleatoria mediante el uso de irradiación (UV) y tratamiento de EMS. De los 40 mutantes examinados *T. dupontii* EMS 15 tenía 2.4 veces más rendimiento (21. 8 U/ml/min) En comparación con la cepa silvestre. También se examinaron los cinco medios de fermentación. El medio 3 resultó en una mayor actividad celulasa (29.3 U/ml/min) y se encontró que era el mejor medio.

Palabras clave: identificación molecular, hongos, CMCase, mutación.

1 Introduction

Cellulose is the major component of plant cell wall. It is unbranched linear polysaccharide made up of glucose subunits attached together through β-1, 4 glyosidic linkages (Das *et al*., 2013). Cellulose is found in two forms including amorphous more susceptible to enzymatic action and crystalline form i.e. highly ordered structure makes it difficult to degrade. Endoglucanase, exoglucanase and β-glucoside are key enzymes of cellulases group and work together during the process of cellulose hydrolysis (Paw *et al*., 2016; Oliveira *et al*., 2019). Fungi are extensively found in nature and have distinctive biochemical pathway to stimulate huge variety of substrates and produce primary as well secondary metabolites like enzyme, antibiotic etc.

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(Keller *et al*., 2005). A vast variety of fungal cultures were screened since decades for the production of enzymes but still majority of them are unexplored. The myriad industrial applications of cellulases create interest in this field. The hydrolysis of cellulosic substrate by fermentation or former processes that could yield glucose as an end product can also help to produce valuable products such as butanol, ethanol etc. Beside these possible applications could include the production of oil, recycling process of paper, pharmaceuticals, fabric bio polishing, look of denim stonewash, deinking of paper and brewages etc. The demand of cellulases is high in contrast to its supply for the bioconversion of lignocellulosic biomass (Wati *et al*., 2007). Therefore, the chance to obtain cheap cellulases will depend on the successful screening of novel cellulose producing strain. Cellulases are produced by large number of microorganisms. Fungi and bacteria are the main natural agents. However, fungi are well known agents for decomposition of organic matter (Lynd *et al*., 2002). Although a large number of fungi can degrade cellulose but only few of them can produce considerable amount of free enzyme possessing the ability to completely hydrolyse crystalline cellulose. Strain improvement by random mutation plays a significant role for better enzyme production as it is cost effective and still reliable. Mutagenic procedures can be optimized in terms of type of mutagen and dose. Therefore, various types of mutagenic techniques such as physical and chemical mutagenesis have been used for cellulase production. Therefore, much research has been aimed in obtaining new microorganisms for cellulase production with higher specific activities and greater efficiency (Rathnan *et al*., 2012).

2 Materials and methods

2.1 Isolation of thermophilic cellulolytic fungi

In order to isolate thermophilic cellulolytic fungi, samples were collected from the compost piles, approx. 10 to 20 cm underground soil from industrial area of Faisalabad, surface litter, grass clippings in garden soil and crop surplus. The cellulolytic fungi were isolated by serial dilution method (Ashwani *et al*., 2014) using boiled chopped filter paper supplemented with mineral nutrients ($CaCl_2$, $MnSO_4$, $NaNO_3$, $MgSO_4$, K_2HPO_4, $FeSO_4$) and agar. The screening of fungal strain for cellulase potential was carried out via submerged fermentation. All isolated fungi were identified morphologically (Salar and Aneja, 2007); while isolated fungal strain which showed highest cellulase activity was further confirmed by molecular characterization.

2.2 Inoculum preparation

3 to 4 days old slant of *Thermomyces dupontii* TK-19 were used for inoculum preparation. 10.0 ml of saline water was added in each slant and without damaging the conidia the medium was slowly scratch and shaken vigorously to get the uniform suspension.

2.3 Submerged fermentation

The 25ml of sterilized fermentation medium was inoculated with 1ml of conidial inoculum (2×10^7 conidia/ml). After inoculation all the flasks were kept on shaking incubator at 40°C for 72 hrs. After 72 hours fermented broth was centrifuged at 9000 rpm for 10 min and supernatant was used for the estimation of cellulase.

2.4 Assay of CMCase

CMCase estimation was carried out according to method given by Gao *et al*. (2008). 0.5 ml of enzyme extract was added in 0.5 ml of 1% CMC prepared in 0.1 M citrate buffer (pH 5) followed by 2 ml of DNS reagent. Blank was also run parallel by replacing the enzyme extract with distill water. The mixture was incubated at 60 ºC for 30 min and reducing sugar was measured at 546nm according to Miller (1959).

One unit of CMCase activity is defined as the amount of enzyme needed to release 1 μmol of glucose from the appropriate substrate per ml per min under standard assay conditions (Gupta *et al*., 2015).

2.5 Estimation of Dry cell mass (DCM)

The Dry cell mass was determined according to Velmurugan *et al*. (2011).

2.6 Random mutagenesis

2.6.1 Physical mutagenesis (UV irradiation)

One ml of conidial suspension prepared from 2days old fungal culture was transferred in Potato dextrose broth. The conidia were allowed to grow for 8 hours at 40°C in shaking incubator at 160 rpm. The medium

(5ml) containing conidia was transferred in sterilized petri plates and subjected to UV irradiation (λ = 283 nm) for 30-120 min. The distance between the sample and UV lamp was kept 10 cm for all the trials to get greater than 95% death rate. In order to keep the stability of thymine-thymine dimmers (T-T) UV treated plates were kept in dark place (Radha *et al*., 2012). All the mutated strains were then subjected to quantitative screening for CMCase production.

2.6.2 Chemical mutagenesis

Chemical mutagenesis was carried out by adding 1.0 ml of 0.1% EMS solution in 1.0 ml of conidial suspension at different time intervals (10-40 min). After treatment, conidial suspension were centrifuged and supernatant was discarded. The pellets were washed thrice with phosphate buffer (pH 7, 0.05 M) in order to remove the traces of EMS. The EMS treated conidia were re-suspended in same buffer (Burlacu *et al*., 2017).

2.7 Fermentation media

Different media were tested for CMCase activity by wild and mutant strain of *T. dupontii*. All the media comprises of g/l. **Medium 1**: $(NH_4)_2SO_4$1.4, KH_2PO_4 2.0, Urea 0.3, $MgSO_4{\times}7H_2O$ 0.3 , $CaCl_2$ 0.3, $FeSO_4{\times}7H_2O$ 0.005, $ZnSO_4{\times}7H_2O$ 0.0014, $MnSO_4{\times}H_2O$ 0.0016, $CoCl_2$ 0.002, Tween 80 2.0 ml, CMC 10.0 (Mandel and Weber, 1969); **Medium 2**: K_2HPO_4, 1; yeast extract, 5; CMC, 10; Czapek's concentration, 10 ml (Coral *et al*., 2002); **Medium 3**: Yeast extract 10, NaCl 2, $CaCl_2$0.2, KH_2PO_4 2, $FeCl_2$ 0.01, $MgSO_4$ 1.7, NH_4Cl_2, wheat bran 5 (Prasanna *et al*., 2016 modified); **Medium 4**: CMC, 1.0; yeast extract, 0.1; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 10; $MgSO_4{\times}7H_2O$, 0.1 and NaCl, 0.2 with 1 g pretreated rice straw (Kocher *et al*., 2008); **Medium 5** Czapek-Dox medium: KCl 0.5, $NaNO_3$ 2, K_2HPO_4 1, sucrose 30, $MgSO_4$ 0.05, $FeSO_4$ 0.01, distilled water 1000 (Prasanna *et al*., 2016).

2.8 Molecular characterization

Molecular characterization of 18SrDNA was carried out according to modified method of Prabha *et al*. (2013).

Extraction of DNA and PCR amplification. For the extraction of DNA 1-2 days old fungal culture was placed over night at -80ºC. Afterward mycelia were grinded in powder form using ice cold pestle and mortar. Preheat 10 ml extraction buffer at 60 ºC before use by addition of 200 μl BME. The 500μl of CTAB extraction buffer (1% CTAB, 100mM Tris-base, 20mM EDTA, 1.4 M NaCl adjusted pH 8.0) was added in fine powder mycelia and vortex for 6 sec. The reaction mixture was than incubated at 80°C for one hour. After this the reaction mixture was centrifuged at 13000 rpm for 1 min.The supernatant was collected in new tube and equal volume of chloroform: phenol (24:1) was added in to it and vortex for 4 sec. The reaction mixture was again centrifuged at 13000 rpm for 3 min. The supernatant was again separated and re-extracted by repeating the previously describe steps. The final supernatant was transferred in another centrifuge tube and 300 μl of chilled isopropanol was added in to it and incubated for 30-60 min at -20°C. After this reaction mixture was centrifuge at 13000 rpm for 5 min. The supernatant was discarded and pellet formed was washed with 70% ice cold ethanol, dried and extracted DNA was re-suspended in 30 μl of sterile water. The ITS region (5.8S rDNA) was amplified using universal primers ITS-1(F): TCCGTAGGTGAACCTGCGG and ITS-4 (R): TCCTCCGCTTATTGATATGC (Kumar *et al*., 2014). Initial denaturation was done at 94 ºC for 5 min followed by 30 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec, 72ºC for 1 min and final extension was done at 72 ºC for 5 min. PCR product was excised and gel purified using gel extraction kit (GeneJET #K0691). PCR products were cloned using InsTAclone^{TM} PCR Cloning Kit (#K1217) and then transformed in DH5α. Positive colonies were sent to Ist base Singapore for sanger sequencing with above mentioned primers.

2.9 Bioinformatics analysis

Sequence was analyzed using BLAST and MEGA7 was used to construct phylogenetic tree (Kumar *et al*., 2016).

2.10 Statistical analysis

All the data were put through statistical analysis in order to find out the significance by using ANOVA in SPSS 16.0 software.

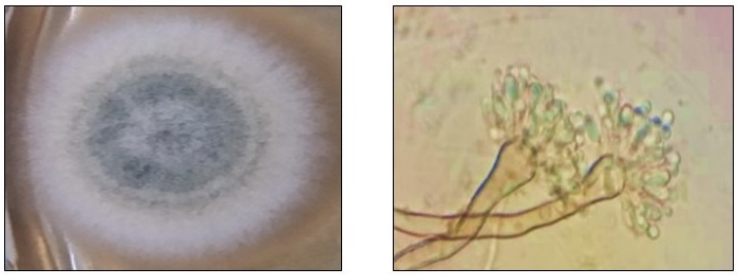

Fig. 1. *Thermomyces dupontii* TK-19 a) Colony on PDA (b) Microscopic structure.

3 Results and discussion

3.1 Isolation, screening and identification of cellulolytic fungi

The exploration of novel fungal strain having potential for targeted product is a very vital step for the success of fermentation process. A large number of fungal strains were isolated and screened during the process of production and optimization of enzyme and it became very essential to understand their characteristics due to several reasons. Morphological and biochemical features of fungi are commonly considered for their identification but distinction of closely related species needed extensive phenotypic as well as molecular investigation (Shahriarinour *et al*., 2011). In the present study 70 fungal strains were isolated and screened.

Out of these isolates 30 were identified as *Aspergillus* sp, 12 as *Trichoderma* sp, 12 as *Thermomyces* sp and 16 were *Penicellium* sp. All the isolates were screened quantitatively for cellulase production. The data of top 8 strains were presented in Table 1. The strain showing highest ability for Carboxy methyl cellulase production was identified morphological as well on molecular basis. Among all the celluolytic strains maximum CMCase production (8.93 U/ml/min) were recorded by *Thermomyces dupontii* and given the code TK-19. Morphological studies of *Thermomyces dupontii* TK-19 indicate the colonies are round margin are white and green or pale green from the interior. Conidiophores were stipe hyaline and smooth with many divergent phialides (Fig. 1a & b). *Thermomyces dupontii* TK-19 was further confirmed by sequencing the ITS region (5.8S rDNA). Similarity was checked by retrieving sequences from GenBank.

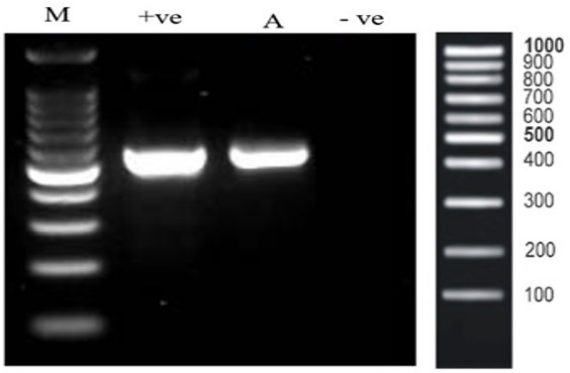

Fig. 2. Isolation of DNA from *T. dupontii* TK-19. M: (DNA Ladder) Marker; +ve: Positive control; A: isolated DNA sample; -ve: PCR with no-template, contains water instead of DNA.

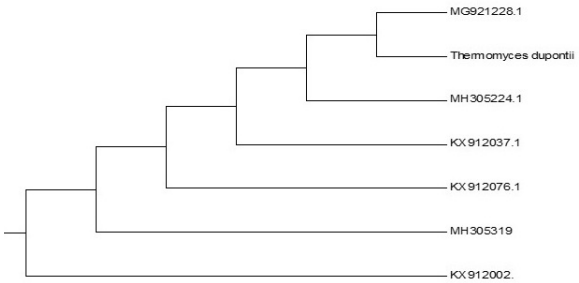

Fig. 3. Phylogenetic tree of *T. dupontii* was computed using the Maximum Composite Likelihood method conducted in MEGA7 showing close similarity to other fungal species.

BLAST analysis showed 98.06% identity with *Thermomyces dupontii*. Maximum Composite Likelihood phylogenetic tree was constructed in MEGA7 (Fig. 3).

Fundamental techniques used to identify the organism generally based on microscopic, morphological approaches that needed more time,

Table 1. Screening of fungi for CMCase (cellulase) production.

Serial No.	Isolates	CMCase activity (U/ml/min)
1	*Aspergillus* sp	5.4±0.2
2	*Aspergillus* sp	7.45±0.1
3	*Thermomyces* sp	**8.93±0.1**
4	*Trichoderma* sp	5.16±0.2
5	*Thermomyces* sp	6.37±0.1
6	*Aspergillus* sp	4.7±0.2
7	*Thermomyces* sp	5.4±0.2
8	*Trichoderma* sp	3.2 ±0.1

labor and knowledge of classical taxonomy. Besides these sometime unreliable results were obtained due to close similarity among the species. Due to limitation in conventional approaches, molecular methods came in use to overcome the identification and classification problems. Amplification of ITS regions of rDNA genes by using specific fungal primers has introduced the possibility of molecular identification of different fungi (Aslam *et al*., 2017). Full ITS region in fungi such as ascomycetes and basidiomycetes has an average length of 500-600 bp (Porter *et al*., 2011). In present research ITS-1 and ITS-4 primers were used for 18S rDNA molecular identification. The fragment size obtained was 550bp (Fig 2). PCR amplicon was cloned in vector and sequences were obtained. The extraction of DNA is most vital step in genomic research. Beside this quantity and purity of extracted DNA plays a key role for further process. The purity and quantity of DNA based on the chemical used in the extraction. In present investigation modified CTAB method was used for DNA extraction. This method was considered best method in contrast to other methods used. CTAB extraction buffer is used as a detergent or strong cationic surfactant which disturbs the cell membrane by binding to the lipids in it. The NaCl used binds to the backbone of DNA and neutralize its charges. The change in the polarity will make the DNA insoluble in water. Some alcohols including isopropanol and ethanol also change the polarity and allow precipitation of DNA. This DNA helps in identification of fungi after further processing through PCR cloning and sequencing. Further sequence was analyzed in BLAST for similarity searches. Mega 7 software was used to construct phylogenetic tree using Maximum Composite Likelihood method. Maximum Likelihood is the method suitable for simple sequences such DNA sequence to build phylogenetic tree. On particular phylogenetic tree, it provides probabilities of given sequence with their evolution (Kumar *et al*., 2016).

3.2 Strain improvement

Strain improvement is the vital step in the development of fermentation process. It provides the means through which production cost is reduced by increasing the productivity. In many cases strain improvement is accomplished by conventional method of mutagenesis. Conventional method of strain improvement by subjecting the microorganism in physical and chemical mutagenic treatment followed by screening and selection of mutant proved to be successful. Although, it is lengthy, labour intensive, trial and error procedure but successful mutation gave enhanced target product (Burlacu *et al*., 2017; Jafari *et al*., 2017). In the present study *Thermomyces dupontii* TK-19 was subjected to both physical and chemical mutagens. The UV treatment results 1.2 fold more CMCase production as compared to wild strain (Table 2). Survival rate was 90 % after 120 minutes exposure to UV irradiations (Fig. 5). Perhaps UV irradiation modified the pyrimidine structure consequently formation of thymine dimer occur which alter organization of DNA double helix and finally stop the replication process and cause mutation. In most cases this leads to better adaptation of microorganism in the environment and show greater CMCase productivity in contrast to parental strain or due to over expression of gene responsible for cellulase production better result was obtained (Hooi and Kuan, 2015) *Thermomyces dupontii* UV-13 was further subjected to EMS treatment and gave 2.4 fold more CMCase productivity in contrast to wild strain (Table 3).When the strain was treated with EMS, 95% death rate was observed after 40 minutes of exposure (Fig. 6).

Table 2. Screening of UV treated isolates for CMCase production.

UV treated isolates	Time of exposure (min)	CMCase activity (U/ml/min)
Wild	0	0
UV -1	30	8.93±0.1
UV- 2		8.73±0.1
UV- 3		8.63±0.2
UV - 4		8.53±0.2
UV - 5		8.92±0.1
UV - 6		8.63±0.2
UV - 7	60	8.73±0.1
UV - 8		8.53±0.2
UV - 9		8.43±0.2
UV - 10		8.92±0.1
UV - 11		8.63±0.2
UV -12		8.73±0.1
UV -13	**90**	**11.3±0.1**
UV - 14		9.3±0.1
UV - 15		6.3±0.1
UV - 16		4.1±0.1
UV - 17	120	2.1±0.2
UV - 18		1.6±0.1
UV - 19		0.8±0.1

Table 3. Screening of EMS treated strains for the CMCase production.

EMS treated isolates	Treatment time (min)	CMCase activity (U/ml/min)
EMS - 1	10	11.2±0.1
EMS - 2		11.3±0.1
EMS - 3		11.4±0.1
EMS - 4		12.3±0.1
EMS - 5		11.9±0.1
EMS - 6		12.1±0.1
EMS -7		11.3±0.1
EMS - 8		11.6±0.1
EMS - 9		11.1±0.1
EMS - 10		11.5±0.1
EMS - 11	20	16.8±0.1
EMS - 12		14.8±0.1
EMS - 13		15.8±0.1
EMS - 14		14.8±0.1
EMS -15	**30**	**21.8±0.1**
EMS -16		19.1±0.1
EMS - 17		11.8±0.1
EMS - 18		8.8±0.2
EMS - 19		5.8±0.2
EMS - 20	40	3.1±0.1
EMS - 21		1.8±0.2

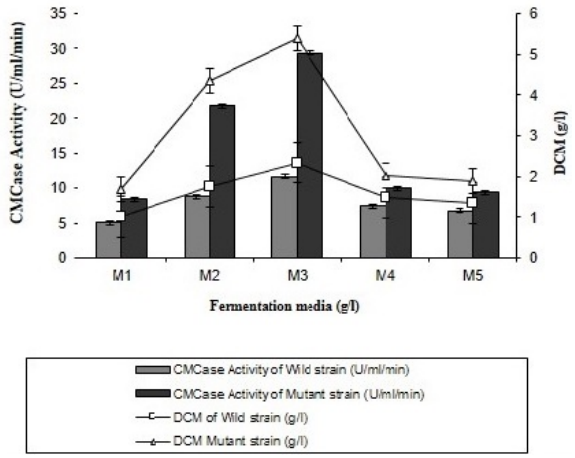

Fig. 4. Screening of different fermentation media for the determination of CMCase.

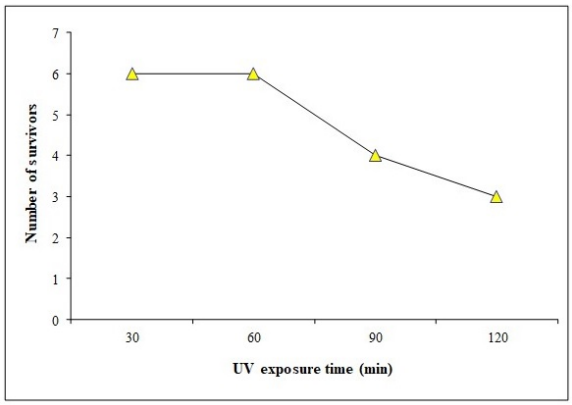

Fig. 5. UV treated survivors of *T. dupontii* TK-19.

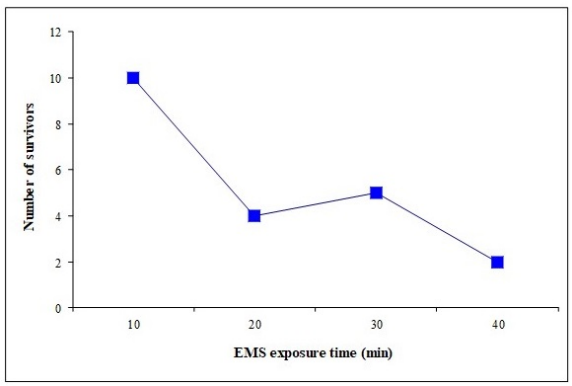

Fig. 6. EMS treated survivors of *T. dupontii* UV-13.

Probably EMS induces mispairing of complementary bases mostly in G/C to A/T transition, and cause alteration in base sequence after replication. This causes permanent changes in the DNA structure or frame shift mutation which leads to enhanced production of CMCase (Shafique *et al*., 2011). Our findings are in accordance with Kumar *et al*. (2015) who reported the effect of UV and EMS on cellulases.

3.3 Screening of different fermentation media

Selection of suitable fermentation medium is one of the significant factors for enzyme production as it provides the energy and nutrients for the microbial growth. The selection of economical, cost effective, simple and easily available medium is essential for biosynthesis of enzymes. In current research five different media were screened for CMCase production by both wild and mutant strain (Fig 4). The M3 medium gave highest CMCase activity (11.7; 29.3 U/ml/min) by both wild and mutant strain respectively, in contrast to all other media. Among all fermentation media maximum CMCase production was recorded in Medium3 this could be reason that wheat bran consist of carbohydrates and proteins. Wheat bran serves as an excellent carbon source due to its large surface area and rich nutritional content such as 27% total carbohydrate, 14% of protein, 64% digestible nitrogen and 6% of lipids (Sharma *et al*., 2016). Our findings are similar to Brito *et al*. (2015) who reported optimal CMCase production by using wheat bran. All other media showed less productivity in comparison with Medium3. This could be due to presence of components having toxic effect on the fungal growth. Some metal ions like Zn^{+2} when used with different concentrations showed adverse effect on fungal growth. Furthermore, replacement of nitrogen source in medium may causes changes in protein synthesis and product formation. The toxin synthesis by the use of zinc on fungal growth in liquid cultures has been reported by Jackson *et al*. (1989). Exposing fungi under different concentration of zinc, magnesium and copper were studied by Mejad *et al*. (2013).

Conclusion

The thermo tolerant *T. dupontii* TK-19 has potential to survive under harsh industrial conditions. The carboxy methyl cellulase produced can be used as a substitute of many chemical processes. Cellulolytic fungal isolates were now preferred for large scale industrial production as compared to bacterial strain.

The mutant *T. dupontii* EMS-15 produce 2.4 fold more CMCase in contrast to wild strain. This newly isolated mutant strain could be potential candidate for industrial cellulase production.

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