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OMICS International has organized 500 conferences, workshops and national symposiums across the major cities including San Francisco, Las Vegas, San Antonio, Omaha, Orlando, Raleigh, Santa Clara, Chicago, Philadelphia, Baltimore, United Kingdom, Valencia, Dubai, Beijing, Hyderabad, Bengaluru and Mumbai.

Isolation, Identification and Evaluation of Highly Cellulases Producing *Trichoderma* Isolates from Egypt

A.I. Fahmi[1], R.A. Eissa[1], K.A. El-Halfawi[2], H.A. Hamza[2] and M.S. Helwa[1]

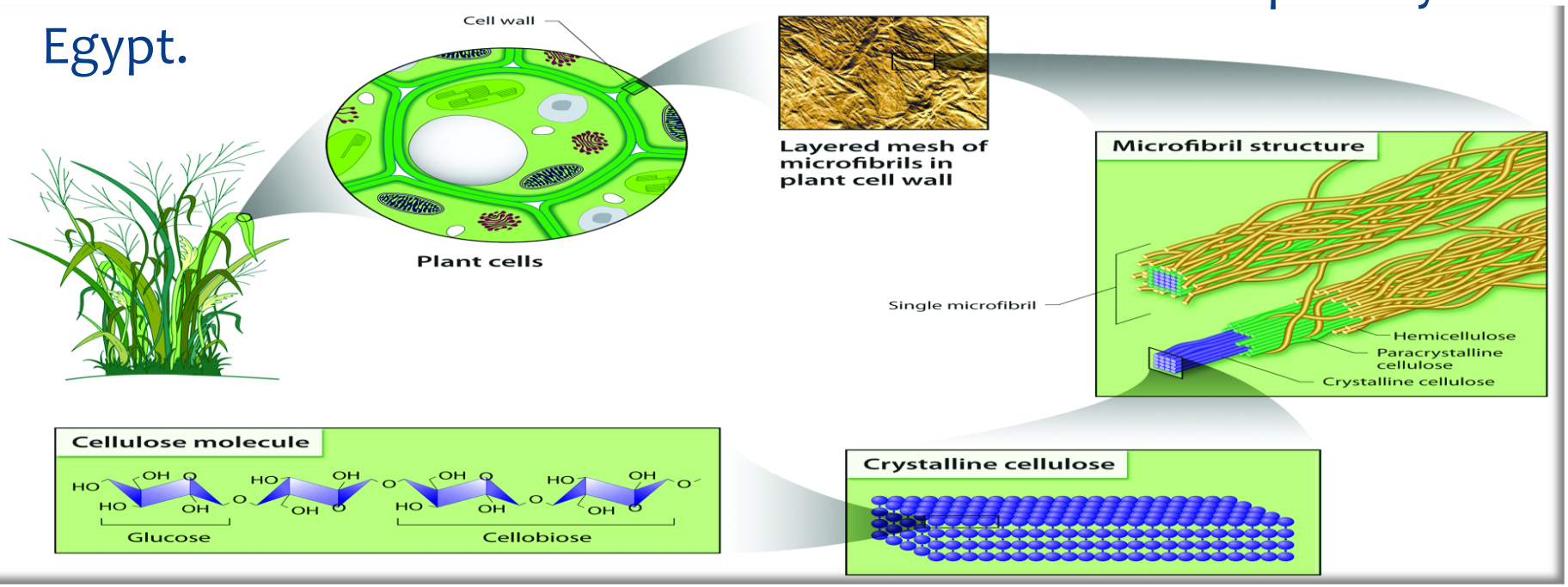
1- Genetics Department, Faculty of Agriculture, Menoufia University
2. Institute of Genetic Engineering and Biotechnology Research, University of Sadat City

Introduction

* Lignocellulosic materials are cheap renewable resources available in large quantities including various agricultural residues, fruit and vegetable wastes, woods, municipal solid wastes, wastes from the pulp and paper industry.

Introduction

* One of these important cellulosic materials is rice straw which is a major agricultural waste in rice-growing countries. Rice straw still a waste till now all over the world and especially in Egypt.



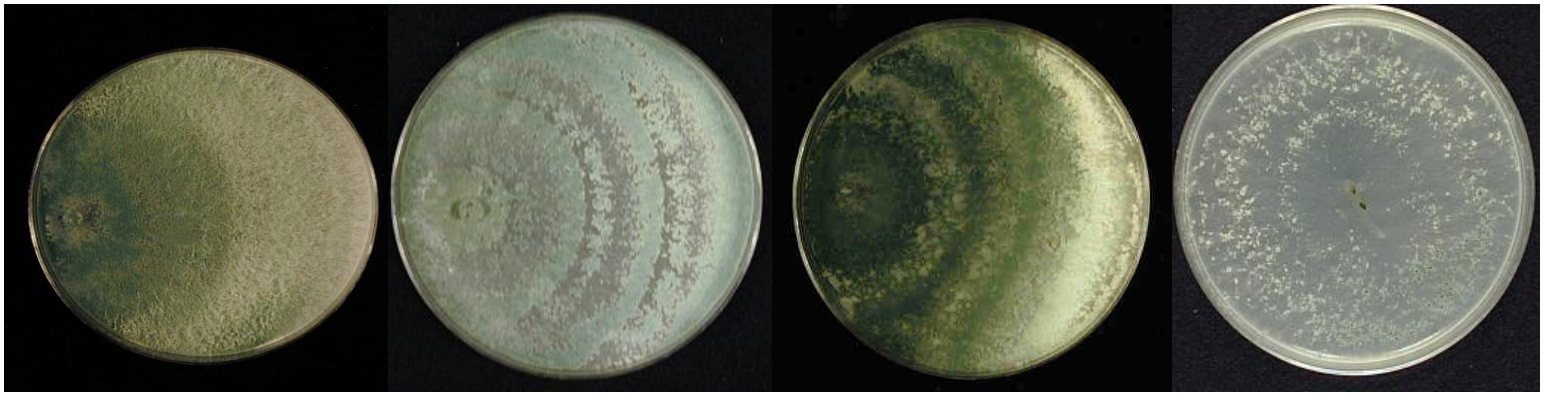
Rice straw viability in comparison with the others four major crops residues in Egypt.

Crop residues	Production (million tons/year)	Current usage	Un utilized amount (million tons/ year)	Un utilized amount (%)
Rice straw	5.0	Few amount for animal fodder and Composting	3.1	62 %
Wheat straw	8.2	Almost totally used as animal fodder	0.082	1 %
Maize residues	6.7	Almost totally used as animal fodder	0.67	10 %
Sugar cane residues	4.8	Used as fuel in sugar factories	0.69	14.4 %
Cotton stalks	1.3	Fuel in rural area	0.65	50 %



Trichoderma Spp. are evolutionary “factories” of cellulolytic enzymes

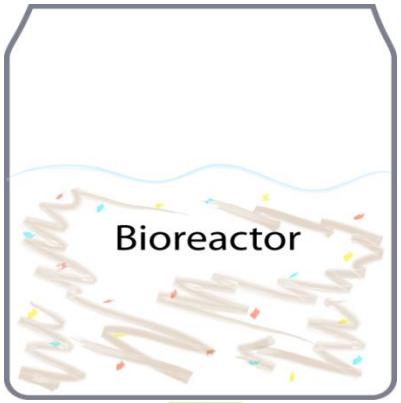


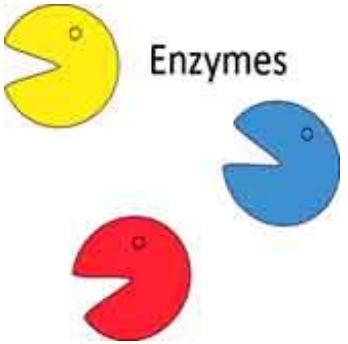


Cellulose, the major fraction of lignocellulosic biomass, can be hydrolyzed to glucose by cellulase enzymes





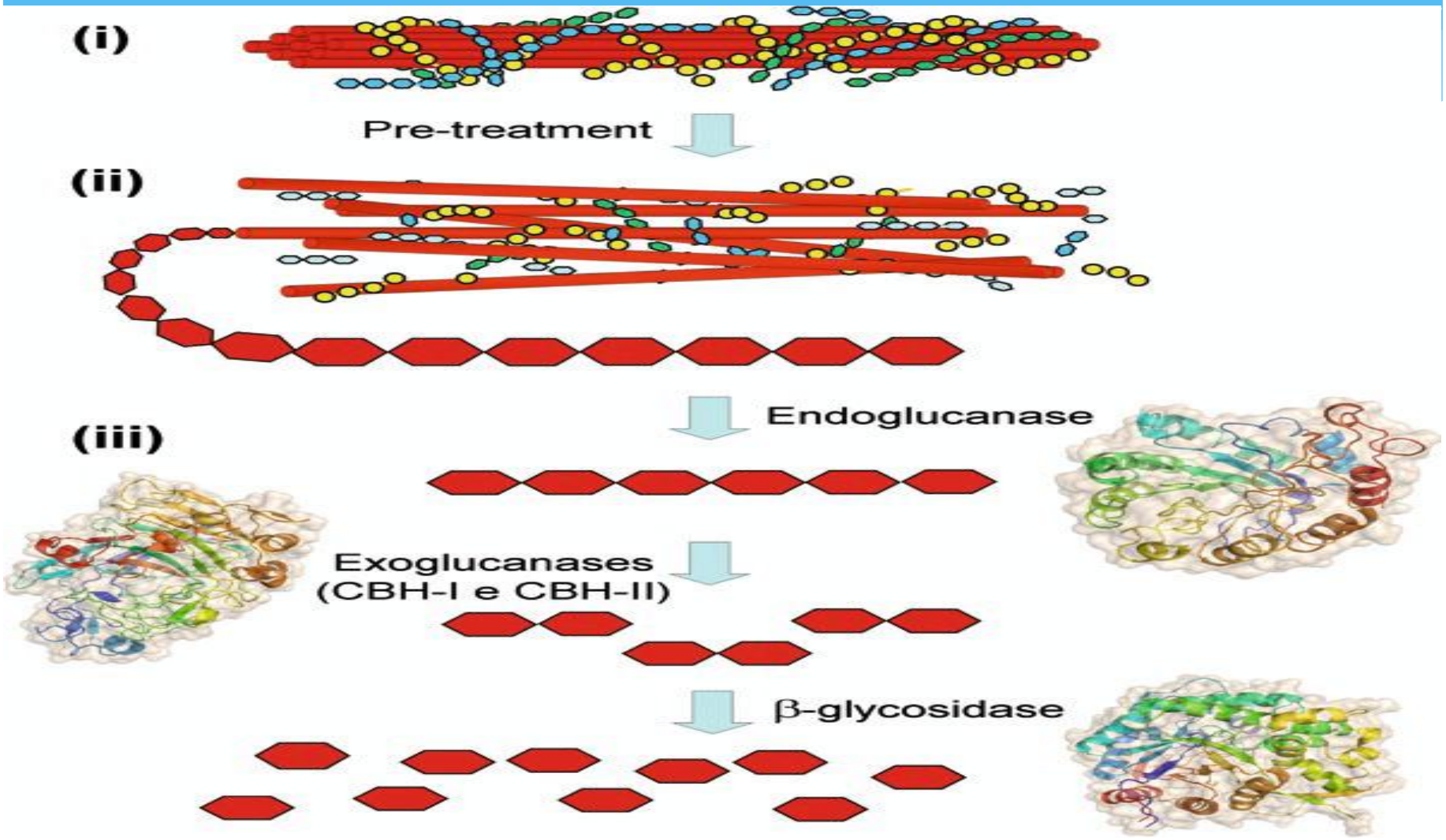




Fermentation / Biofuel

Chemical industry

Cellulases are enzymatic complex, that comprises exo-β-1,4-glucanases (EC 3.2.1.91), endo- β -1,4-glucanases (EC 3.2.1.4) and β -1,4-glucanases (EC 3.2.1.21), that act synergistically in the hydrolysis of β-1,4-glycosidic bonds present in cellulose polymers



Objectives

1) Characterization of some *Trichoderma* isolates morphologically and molecularly.

2) Evaluation and selection of the best isolates for producing cellulases enzymes.

Samples collection

* Samples were Collected from six governorates of Egypt

*** Samples type :**

- Decomposed rice straw
- Decomposed Wheat straw
- Decomposed tree leaves
- Garden soil
- Field soil

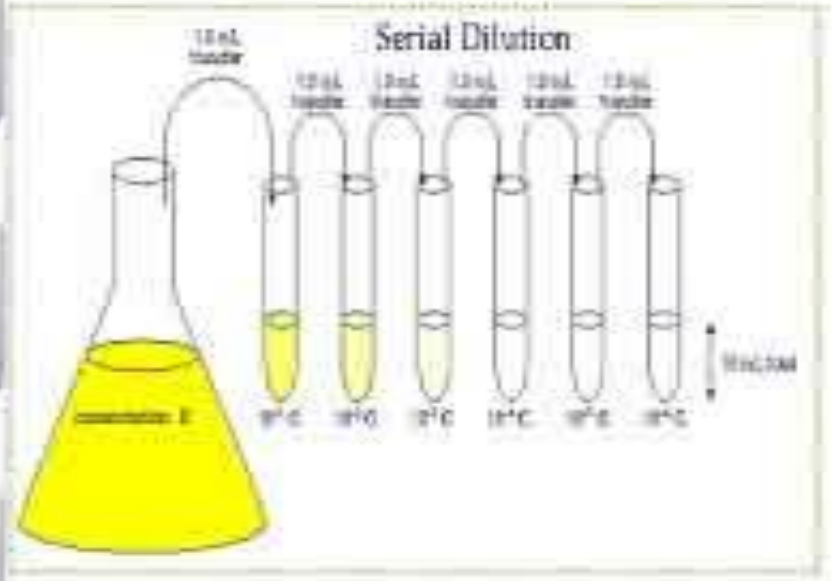
Isolation of *Trichoderma* sp.

It was made through a serial dilution technique on PDA media.

20 gm of soil of each collected were measured

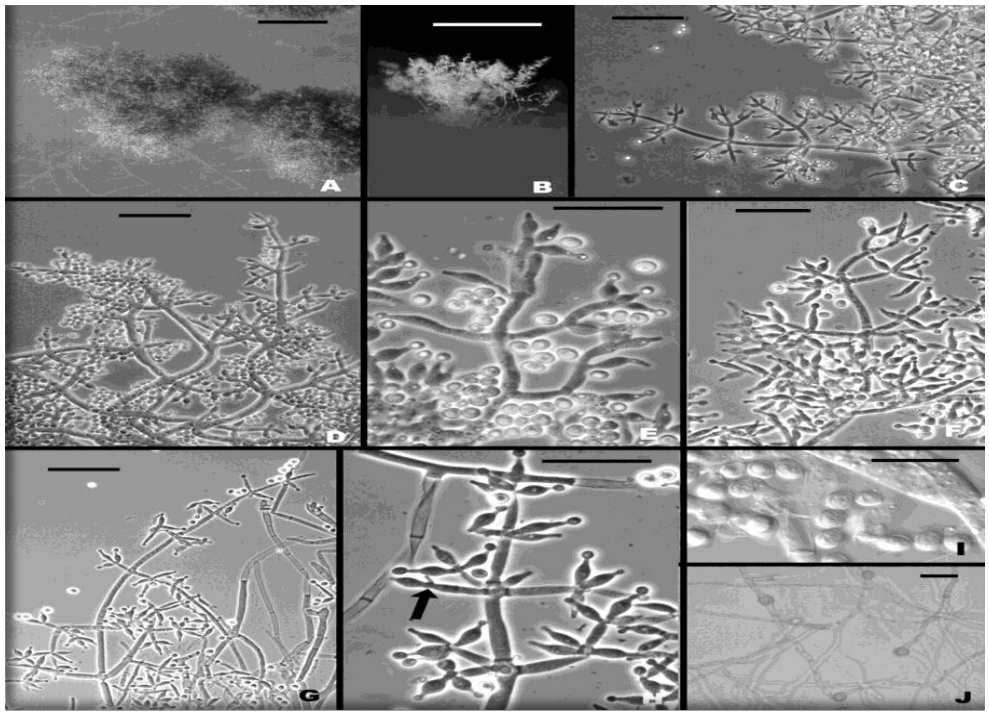
20 gm of soil sample were taken into 250 ml conical flask containing 100 ml of sterile physiological saline

Each conical flask of samples was vortexed vigorously for 15 min



Morphological Characterization of 27 isolates

based on conidiophore branching pattern and conidium morphology key provided by Rifai (1969), Barnett (1998) and Bissett (1991)

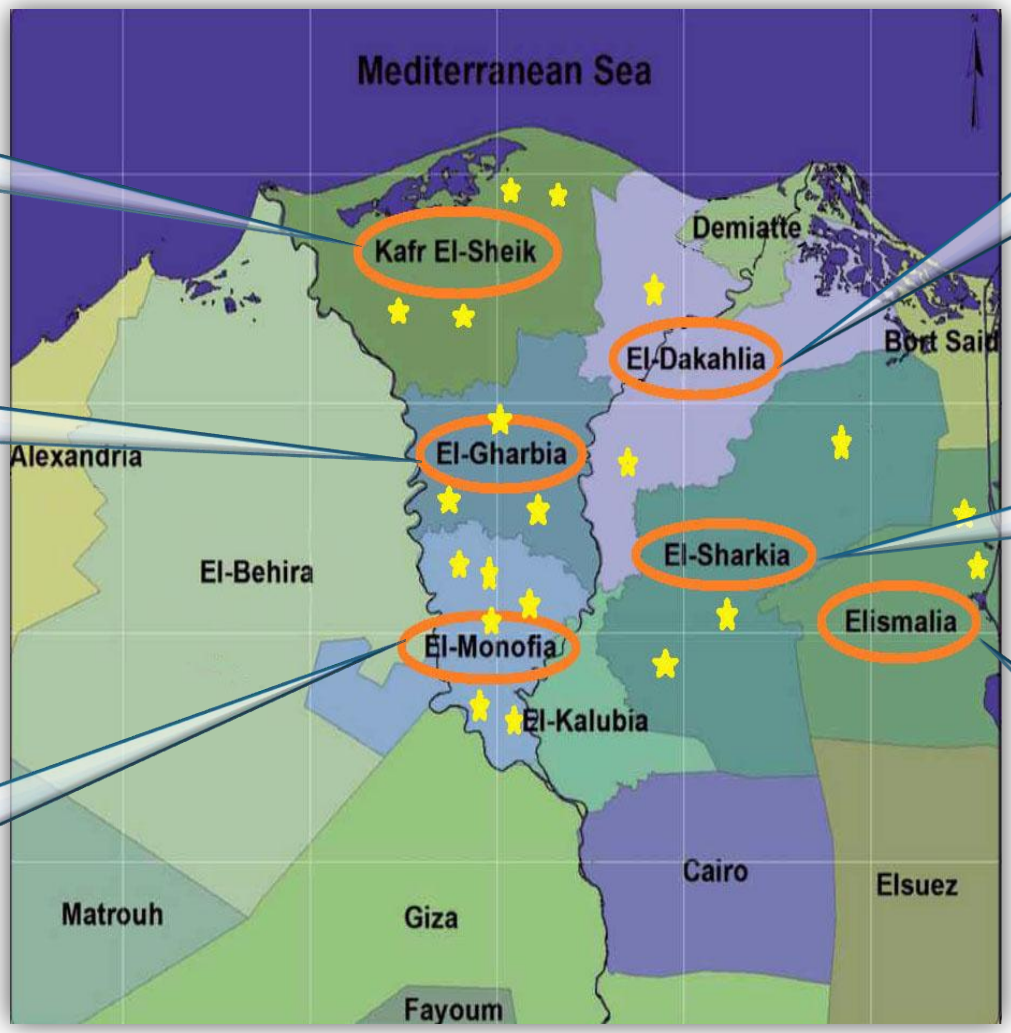







Mediterranean
Port Said
Alexandria
Suez Canal
Qattara Depression
Cairo
Siwa Oasis
Sinai
Al Minyah
Eastern Desert
Asyut
Western Desert
Luxor
EGYPT
Aswan
Lake Nasser

Egypt



T. harzianum	T10
T. asperellum	T31
T. Koningii	T20
T. harzianum	T32

T. harzianum	T24
T. viride	T25

T. koningii	T3
T. hamatum	T19
T. harzianum	T42

T. harzianum	T14
T. reesei	T17
T. harzianum	T40

T. harzianum	T1
T. Viride	T2
T. hamatum	T18
T. harzianum	T21
T. harzianum	T41
T. hamatum	T44

T. viride	T26
T. koningii	T43

Nile delta of Egypt

Molecular identification of 11 isolates

Analysis of the internal transcribed spacer (ITS) 1 and 2 of ribosomal DNA (rDNA)

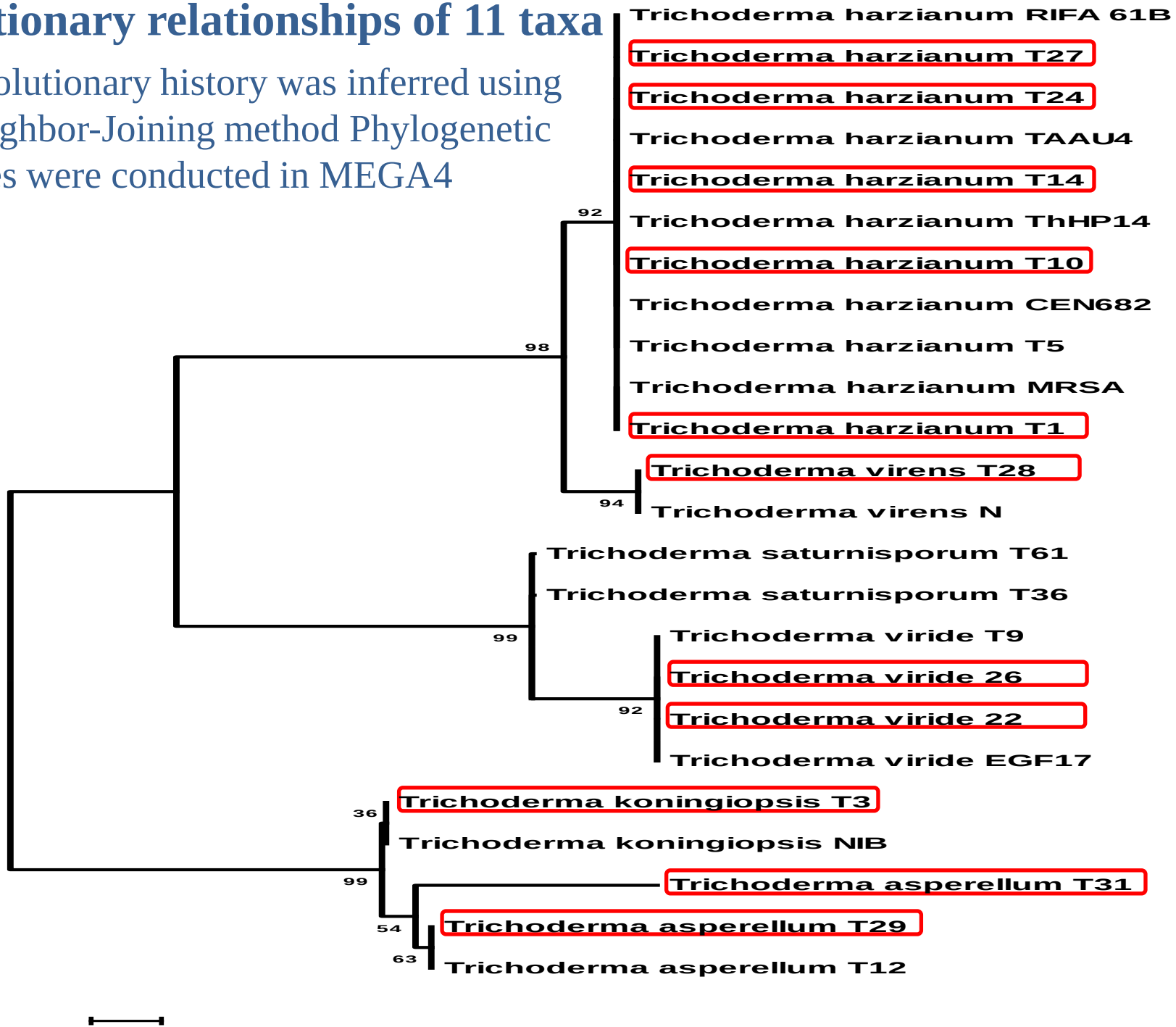
1. DNA extraction
2. PCR amplification of ITS1-5.8S- ITS2 rDNA region using the primer pair: ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3')
   ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3')
3. Sequencing of amplified DNA fragments
4. The sequences of ITS1-5.8S-ITS2 regions were manually aligned using Molecular Evolutionary Genetics Analysis (MEGA4 version 5.10.)
5. The sequencing data were compared against the Gene Bank database (http://www.ncbi.nlm.nih.gov/BLAST/)
6. Sequences were will submitted to GenBank

Some isolates used in this study and the accession of the aligned species for ITS1-5.8S-ITS2 sequence in GenBank

Isolate	Alignments Description	Max score	Query coverage	E value	Max identification	Accession
T. harzianum (T1)	Trichoderma harzianum MRSA	1022	100 %	0.0	99 %	HG008049.1
T. koningiopsis (T3)	Trichoderma koningiopsis NIB	992	100 %	0.0	99 %	KM246760.1
T. Harzianum (T10)	Trichoderma harzianum ThHP14	680	100 %	0.0	100 %	KP064225.1
T. harzianum (T14)	Trichoderma harzianum TAAU4	1003	100 %	0.0	100 %	KM875463.1
T. harzianum (T24)	Trichoderma harzianum RIFA 61B	1009	100 %	0.0	100 %	KF624792.1
T. Viride (T26)	Trichoderma viride T9	1046	98 %	0.0	100 %	HQ259986.1
T. harzianum (T27)	Trichoderma harzianum RIFA 61B	1009	100 %	0.0	100 %	KF624792.1
T. Virens (T28)	Trichoderma virens N	1005	100 %	0.0	100 %	KP671477.1
T. Asperellum (T29)	Trichoderma asperellum T12	989	99 %	0.0	100 %	KP671477.1
T. Asperellum (T31)	Trichoderma asperellum T12	989	99 %	0.0	100 %	KP671477.1
T. viride (T22)	Trichoderma viride EGF17	1036	100 %	0.0	100 %	KJ406563.1

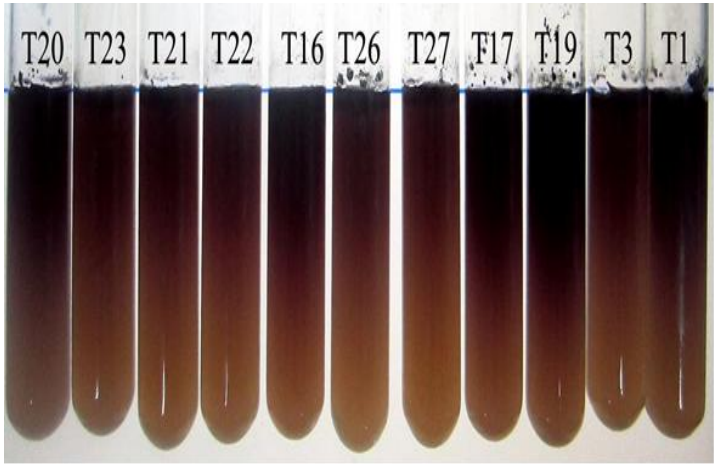
Evolutionary relationships of 11 taxa

The evolutionary history was inferred using the Neighbor-Joining method Phylogenetic analyses were conducted in MEGA4

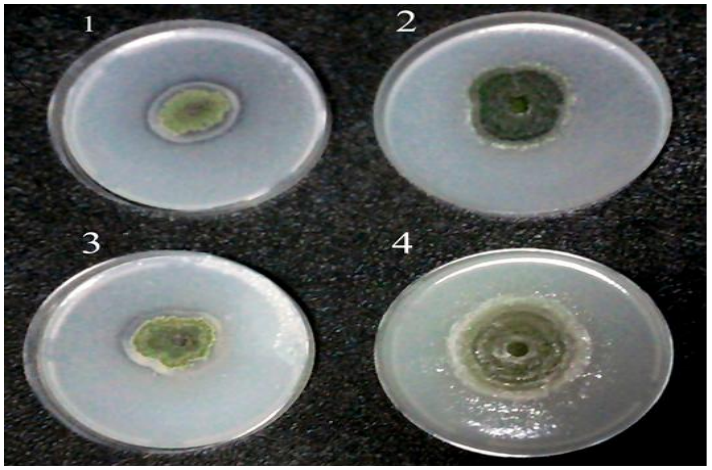


Screening of *Trichoderma* isolates for cellulolytic activity

1. Cellulose azure agar test
2. Plate-clearing assay:
    a. Swollen cellulose
    b. Avicel
3. Dye staining of carboxymethylcellulose agar (CMC agar test)



Changes of color cellulose-azure assays



clear zones around fungal colonies

Plate-clearing assay with Phosphoric acid-swollen cellulose and avicel as the sole source of carbon, Dye staining of carboxymethylcellulose agar (CMC agar) and Intensity of blue azure dye released by growen on cellulose- azure agar medium for 27 *Trichoderma* isolates

Trichoderma isolates	Clear zones diameter (cm)		Yellow zone	Cellulose
	Swollen cellulose	Avicel	CMC	Azure
T.1	5.30 ^{a}	5.43 ^{a}	5.33 ^{fgh}	9
T.2	4.83 ^{bcd}	4.63 ^{cdef}	5.40 ^{fg}	10
T.3	4.20 ^{g}	3.73 ^{ij}	4.97 ^{ghij}	8
T.10	2.06 ^{k}	1.73 ^{lmn}	5.97 ^{de}	9
T.14	4.77 ^{cde}	4.63 ^{cdef}	6.67 ^{ab}	10
T.16	4.67 ^{cdef}	3.73 ^{ij}	4.17 ^{l}	9
T.17	3.73 ^{hi}	5.03 ^{abc}	6.50 ^{abc}	10
T.18	5.10 ^{abc}	5.00 ^{abcd}	6.30 ^{bcd}	10
T.19	4.80 ^{cde}	4.73 ^{cdef}	6.70 ^{ab}	10
T.20	4.23 ^{fg}	4.47 ^{efg}	6.67 ^{ab}	10
T.21	3.60 ^{hi}	3.33 ^{j}	3.20 ^{m}	8
T.22	3.57 ^{i}	3.97 ^{hi}	5.63 ^{ef}	8
T.23	4.37 ^{efg}	4.50 ^{ef}	6.20 ^{cd}	9
T.24	5.40 ^{a}	5.45 ^{a}	6.53 ^{abc}	10
T.25	2.07 ^{k}	1.73 ^{lmn}	5.10 ^{ghi}	8
T.26	4.20 ^{g}	1.30 ^{n}	6.17 ^{cd}	7
T.27	2.00 ^{k}	1.97 ^{kl}	5.20 ^{fghi}	7
T.28	2.00 ^{k}	3.77 ^{ij}	4.37 ^{kl}	8
T.29	4.43 ^{defg}	4.65 ^{def}	6.00 ^{de}	10
T.30	2.00 ^{k}	1.8 ^{lm}	2.80 ^{m}	8
T.31	4.03 ^{gh}	4.03 ^{ghi}	6.77 ^{a}	10
T.32	5.10 ^{abc}	5.23 ^{ab}	4.93 ^{hij}	10
T.40	3.00 ^{j}	1.37 ^{mn}	4.27 ^{l}	9
T.41	1.36 ^{l}	2.27 ^{k}	6.03 ^{de}	7
T.42	4.70 ^{cde}	4.33 ^{fgh}	5.10 ^{ghi}	8
T.43	5.27 ^{ab}	5.3 ^{a}	4.53 ^{jkl}	10

Tricoderma isolates that showed high cellolytic ability.

Trichoderma code	Species name
T1	Trichoderma harzianum
T14	Trichoderma harzianum
T17	Trichoderma reesei
T18	Trichoderma hamatum
T19	Trichoderma hamatum
T20	Trichoderma koningii
T24	Trichoderma harzianum
T31	Trichoderma asperellum
T32	Trichoderma harzianum
T43	Trichoderma koningii
T44	Trichoderma hamatum

Screening of *Trichoderma* isolates for cellulase production

1. Cellulase production by Submerged fermentation **(SMF)**.

2. Cellulase production by Solid state fermentation **(SSF)**.

Cellulase production by Submerged fermentation (SMF).

- The submerged cultivation was carried out in 250ml flasks containing 100ml of Mandel's medium with microcrystalline cellulose powder which was used as the sole source of carbon at a concentration of 1%.

- Flasks were inoculated with conidial suspension to provide a final concentration of 1×10^6 conidia per ml and incubated with agitation (160 rpm), at 28 °C, for 6 days.

Determining of cellulase activity

1. Total cellulase activity by FPase activity (DNS method) .
2. Activity of individual cellulases:
   a. β-Glucosidase using cellobiose substrate (GOD method).
   b. Carboxymethyl cellulase (CMCase) using CMC substrate.
3. Free sugar determination in culture filtrate.
4. Total protein concentration determination in culture filtrate.

Enzyme activities and extracellular protein of *Trichoderma* isolate produced in submerged fermentation (SMF) cultures

Isolates	Enzymes activities (IU/ml)			Free sugar (mg/ml)	Total Protein (mg/ml)
	FPase	CMCase	β-glucosidase		
T1	0.29 ^{i}	0.86 ^{fg}	0.14 ^{b}	0.01 ^{i}	0.18 ^{cd}
T14	0.40 ^{g}	0.83 ^{gh}	0.09 ^{c}	0.01 ^{i}	0.30 ^{a}
T17	0.34 ^{h}	1.09 ^{d}	0.02 ^{d}	1.82 ^{b}	0.05 ^{e}
T18	0.59 ^{d}	1.18 ^{c}	0.12 ^{b}	1.77 ^{c}	0.21 ^{be}
T19	0.64 ^{c}	0.88 ^{f}	0.00 ^{d}	0.20 ^{h}	0.33 ^{a}
T20	0.51 ^{e}	0.81 ^{h}	0.07 ^{c}	0.19 ^{h}	0.09 ^{e}
T24	0.85 ^{b}	1.28 ^{b}	0.25 ^{a}	0.43 ^{g}	0.14 ^{d}
T31	0.46 ^{f}	0.74 ^{i}	0.13 ^{b}	2.95 ^{a}	0.18 ^{cd}
T32	0.66 ^{c}	1.21 ^{c}	0.01 ^{d}	0.87 ^{f}	0.14 ^{d}
T43	0.57 ^{d}	1.32 ^{a}	0.01 ^{d}	1.17 ^{e}	0.23 ^{b}
T44	1.17 ^{a}	1.01 ^{e}	0.09 ^{c}	1.59 ^{d}	0.32 ^{a}

Enzyme specific activities of *Trichoderma* isolate produced in submerged fermentation (SMF) cultures.

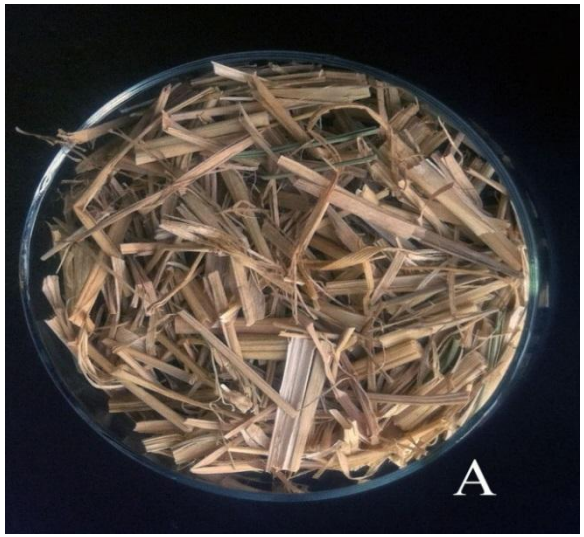
Isolates	Specific activity (Umg^{-1} protein)		
	FPase	CMCase	β-glucosidase
T1	1.63 ^{j}	4.80 ^{e}	0.79 ^{b}
T14	1.32 ^{k}	2.77 ^{h}	0.29 ^{f}
T17	6.87 ^{a}	21.72 ^{a}	0.36 ^{e}
T18	2.81 ^{f}	5.61 ^{d}	0.59 ^{d}
T19	1.95 ^{i}	2.67 ^{h}	0.002 ^{i}
T20	5.69 ^{c}	9.03 ^{b}	0.74 ^{c}
T24	6.06 ^{a}	9.15 ^{b}	1.81 ^{a}
T31	2.53 ^{g}	4.13 ^{f}	0.72 ^{c}
T32	4.72 ^{d}	8.67 ^{c}	0.10 ^{g}
T43	2.47 ^{h}	5.73 ^{d}	0.05 ^{h}
T44	3.67 ^{e}	3.15 ^{g}	0.27 ^{f}

Cellulase production by Solid state fermentation (SSF).

- Pretreatment of rice straw.
- Solid-state fermentation cultures.

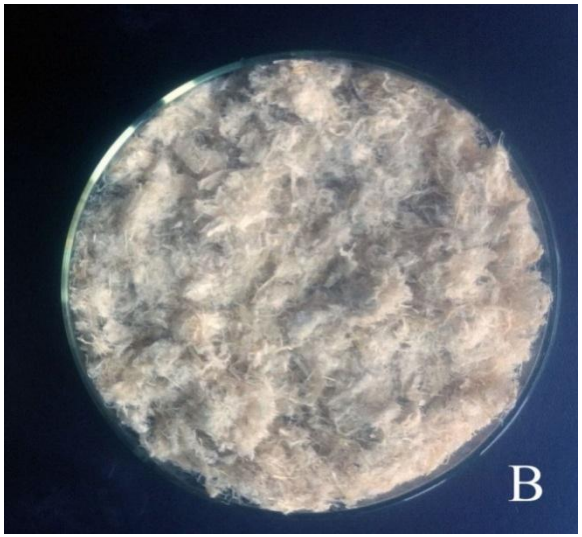
• Pretreatment of rice straw:

Rice straw was pretreated with Microwave and alkali according to the method of Zhu et al. (2005)



Alkali Treatment:
1% NaOH

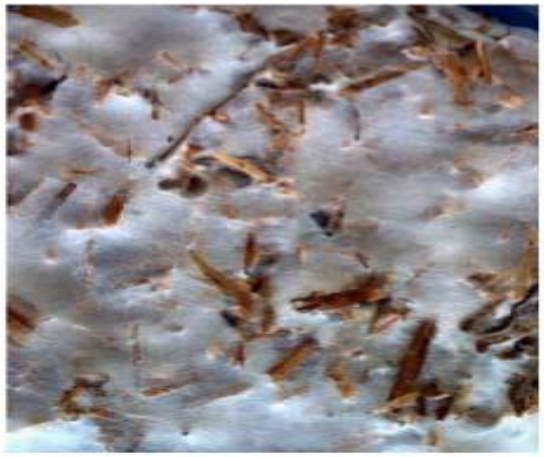
Microwave Treatment :
Frequency : 2450 MHz
Irradiation power: 700 W
Treatment time: 30 min.

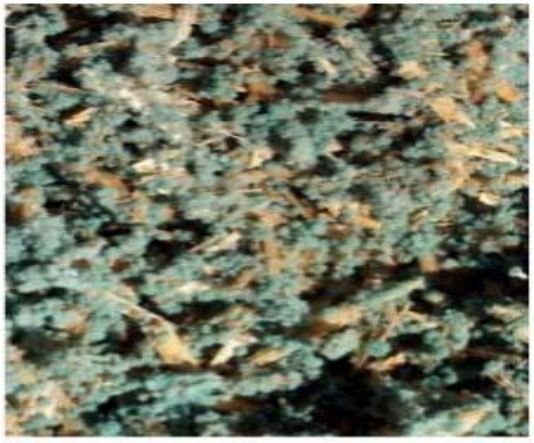


Before		After
38.8 %	Cellulose	↑ 69.2 %
20 %	Hemicellulose	↓ 10.2 %
13.6 %	Lignin	↓ 4.9 %

Solid-State fermentation cultures.

- The solid-state culturing was performed in 250 ml flasks.
- Each flask contained 3 g of mass of pretreated rice straw and 12 ml basal salt solution (MS) 80% humidity.
- autoclaved at 121°C for 30 min.
- Each flask was then inoculated with a final concentration of 1×10^7 conidia per gram of pretreated rice straw.
- The flasks were incubated at 30 °C with a relative humidity of 90% for 12 days.





Extraction of the enzymes after incubation

- 50 ml of distilled water were added, mixed.
- incubated under agitation for 1h at 30 ◦C, at 150 rpm.
- filtered, using dampened cheese cloth.
- The filtrates were centrifuged at 5000 rpm (4◦C) for 15 min to remove spores of the organism.
- the clear supernatant were used as crude enzyme extracts for enzyme assay.
- The filtrate was stored at 4 ◦C until assay for enzymes activities.

Determining of cellulase activity

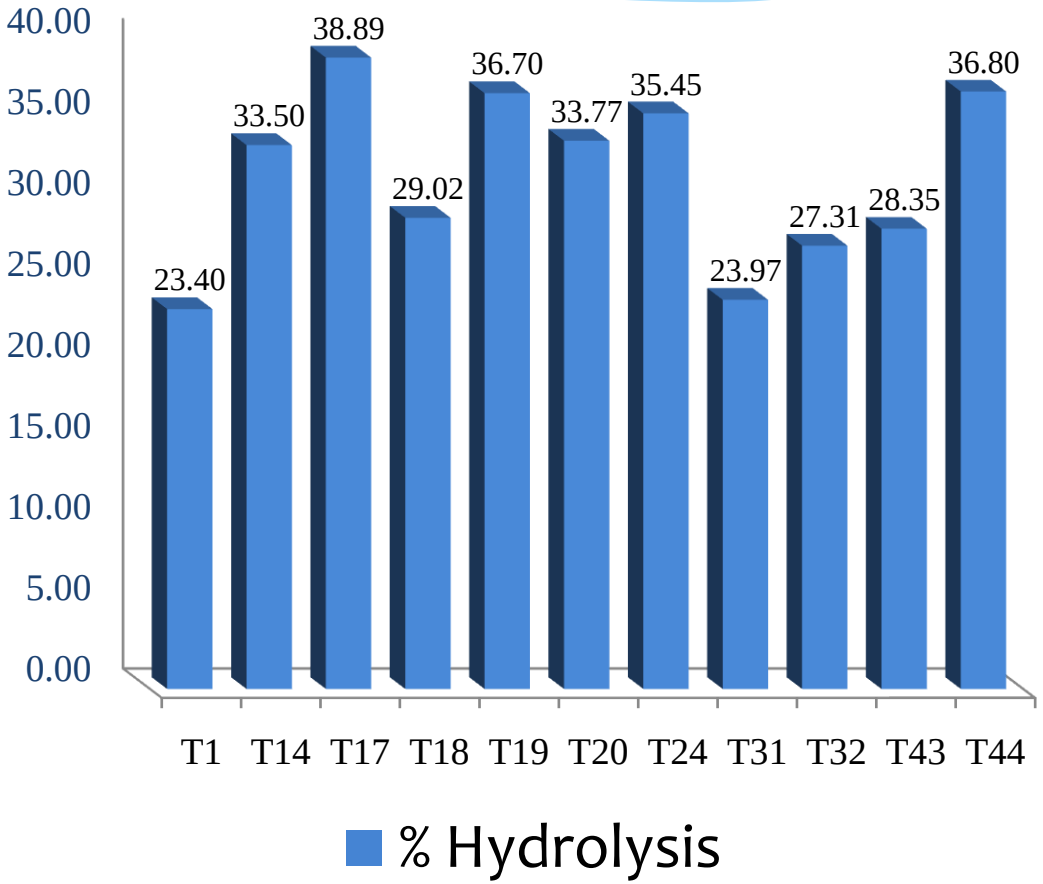
1. Total cellulase activity by FPase activity (DNS method) .
2. Activity of individual cellulases:
   a. β-Glucosidase using cellobiose substrate (GOD method).
   b. Carboxymethyl cellulase (CMCase) using CMC substrate.
3. Free sugar determination in culture filtrate.

Enzyme activities and extracellular protein of *Trichoderma* isolate produced in solid state fermentation (SSF) cultures.

Isolates	Enzymes activities (IU/g)			Free sugar (mg/g)
	FPase	CMCase	β-glucosidase	
T1	0.04 h	2.40 d	0.55 e	15.29 h
T14	1.75 a	2.45 cd	3.56 a	38.5 e
T17	0.48 e	2.51 c	0.08 i	62.06 b
T18	1.55 b	8.44 a	0.33 g	37.82 e
T19	1.12 c	0.18 j	0.83 cd	49.67 c
T20	1.53 b	0.30 i	0.90 c	86.89 a
T24	0.71 d	1.04 g	0.76 d	35.34 f
T31	0.13 g	1.89 e	0.22 h	16.6 h
T32	0.38 f	1.75 f	0.43 f	19.26 g
T43	0.33 f	3.50 b	0.46 f	4.073 i
T44	0.53 e	0.79 h	1.05 b	45.57 d

Hydrolysis percentage of treated rice straw in solid state fermentation by *Tricoderma* isolates.

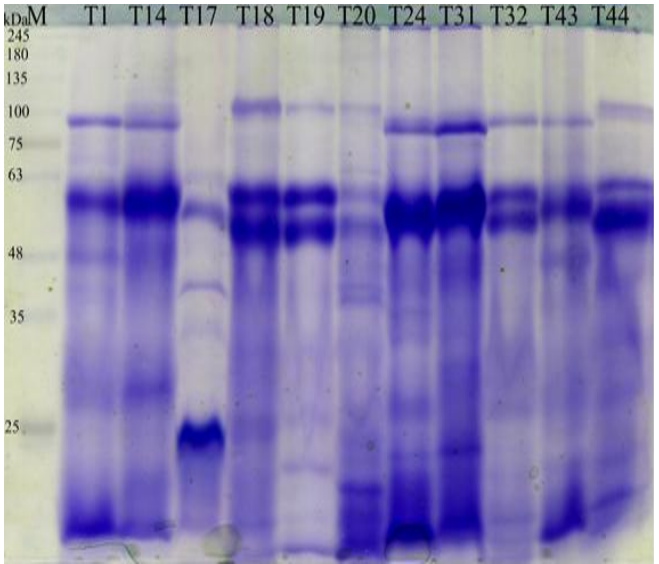
1. The residues of SSF were collected by filtration and washed extensively with distilled water.
2. dried at 65°C to maintain constant weight and weighed .
3. calculation of hydrolysis percent for RS.



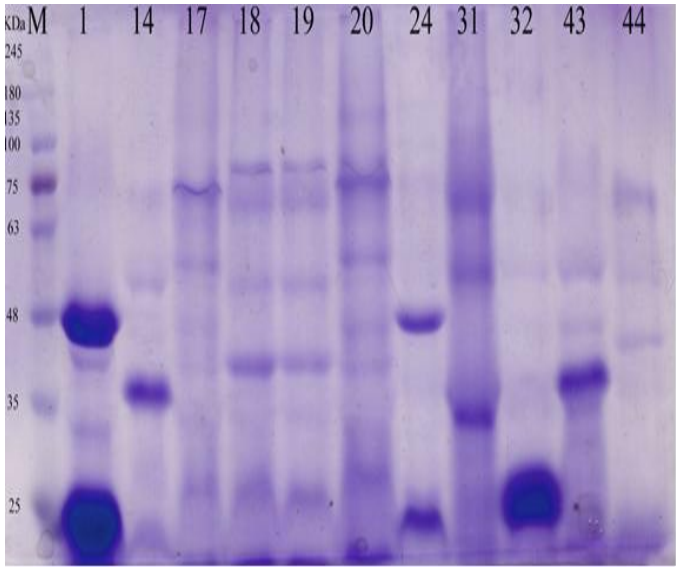
Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis

Extracellular protein of*Trichoderma* isolates that grown in solid-state cultures and submerged cultures were recovered from culture supernatant and analyzed by electrophoresis in denaturing conditions

- **M, maker (in kDa)**
- **lane 1-12 crude protein of (SMF)**



- **M, maker (in kDa)**
- **lane 1-12 crude protein of (SSF)**



Conclusion

- T14, T17, T19, T24, T31and T44 isolates were the highest for producing cellulases enzymes.
- They could be recommended for biotechnological applications.



THANK YOU



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