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Improving of cellulase productivity of *Trichoderma reesei* isolate by using physical and chemical mutagens

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Abstract: Seven cellulytic fungal isolates were achieved referred to Trichoderma species isolated from different soils from ancient town Ayutthaya in Bangkok/ Thailand. The isolates were subjected for two screening methods primary and secondary screening to select the most cellulases productivity. From screening results, it cleared that the isolate which marked (T5) was the best. Cultural and microscopial identification tests showed that it refers to *Trichoderma reesei*. The UV light mutant isolate for 20 min. which marked (Tr-UV 20) achieved a high productivity of cellulase which was 17.38U/ml. while the mutation operation by Gamma ray (2KG) showed increasing in production ability for the isolate marked (Tr2-G2) which was 28.55U/ml. Using Nitrosoguanidine achieved great success in increasing cellulase productivity which was 42.26U/ml for the mutant isolate marked (Tr-NTG3), it was 5 times of their parents.

Keywords: Cellulose, Gamma ray, *Trichoderma reesei*,

INTRODUCTION

Cellulose is one of the abundant chemical compounds on the earth for its existence in plant tissues. It is non crystalized material, non-soluble in water, composed of linear homogenized poly saccharides of glucose unit conjugate with each other by β-1,4- Glucosydic linkage, it could be transferred to a usable

material by degrading these linkages by cellulases which work to analyze cellulose to simple saccharide that microorganisms could use it industrial fermentations to produce different useful materials such as ethanol and single protein (11, 28, 21). Cellulases are group of enzymes (Endoglucanase, Exoglucanase and β-glucosidase) work together to analyze cellulose. In spite of possession many organisms these enzymes but the most important source is microorganisms specifically fungi (10, 16) which acquire a great interest and studies more than that produced by other groups of microorganisms (25, 22). Generally cellulases used in detergent and tissues industry, pulp making and biofuel production (8, 24, 27). The world market estimate about 1.6 billion Dollars for enzyme production, 56% of them for industrial technology, and there are three companies around the world controlled enzyme production, Novo Nordisk 44%, Gerencor International 21% and DSNN.V. 18%, and there are little producers and distributers in North America, Europe and Japan and remain 27% belong to China (30).

Improving fungal strains productivity for industrial production is an important step for all industrial fermentation for reducing production costs and ability to gain desire properties, and because microorganisms produce little amounts of enzyme which make them with no economic benefit (23). So the importance of bio industrial is cleared here which achieved a big success in increasing these products by genetic engineering using mutation as one of genetic vibration source. The aim of this study is to improve cellulase productivity of fungal isolate by using physical and chemical mutagens.

MATERIALS AND METHODS

Experiments were executed at biomass unit/science College / Chulalongkorn University/ Bangkok/ Thailand. The experiment of mutation by Gamma ray was executed at Biotechnology Center/Wyatt Technology Corporation, Santa Barbara, CA.

Samples collection: Soil samples were taken from 15 cm in depth after removing 5 cm of soil surface from old ancient Ayuthaya city in Bangkok. Samples were kept in polyethylene bags until using.

Isolation method: Fungal isolates achieved by serial dilution method according to (15).

Culture media:

1. Potato Dextrose Agar (PDA): used for activation and storage the isolates.
2. Primary screening media: This media was used for detection of fungal isolate ability to produce cellulases by observing clear zone diameter, this media prepared according to the method mentioned by (37) which contains: Carboxy methyl Cellulose (CMC)-10g, $MgSO_4.7H_2O$-0.5g, $NaNO_3$-2g, KCL-0.5g, K_2HPO_4-1g, Agar-20g, pH =6.0.
3. Cellulase production media: prepared according to the method mentioned by (17) which contains: Carboymethyle Cellulose (CMC)-10g, $NaNO_3$-2g, K_2HPO_4-1g, $MgSO_4$-0.5g, KCL-0.5g, $CuSO_4.7H_2O$-0.005g, $MnSO_4.7H_2O$-0.0016g, $ZnSO_4.7H_2O$-0.0014g, $COCL_2.6H_2O$-0.002g.
4. Rose Bengal media: A special media for Trichoderma species prepared according to the method mentioned by (2) which contains: Glucose-3.0g, NH_4NO_3-1.0g, $MgSO_4.7H_2O$-0.2g, K_2HPO_4-0.9g, KCL-0.15g, Chloromphenicol-0.25g,Rose-bengal-0.15g, Agar-20g, pH =6.0.

5. Mutant isolation media: prepared according to the method mentioned by (18) that contains: cellulose-10g, Urea-0.3g, $(NH_4)_2SO_4$-1.4g, KH_2PO_4-2.0g, $CaCl_2$- 0.3g, $MgSO_4$-0.3g, yeast extract-0.25g and peptone 0.75g and agar-17.5g.

Spore suspension: It is prepared by adding 5 ml of 1% sterilized Tween 80 to fungal culture growth in test tubes contain PDA media. Shaking tubes and scraped the culture to gain spores. 1ml of this suspension was added to 9ml of 0.85% NaCl solution, and from this spore suspension serial dilutions were prepared and the count was done by Haemocytometer (34). The spore suspension was added to production media with ratio of 4%, incubated in shaker incubator on 28±2°C with 150 rpm for 5 days.

Identification of the isolate was done according to identification keys mentioned in (4, 26).

Detection of cellulase production ability: the detection was done by cutting a disc of grew fungal colony by needle to a petri dish contains detection media, and then incubated on 28±2°C for 5 days. The red Congo dye 0.1% was prepared, and then added to the petri dish for 10 min., washed by (1N) NaCl for 15 min. many times. Cellulase production ability of fungal isolate was calculated according to the equation mentioned by (29):

Cellulase production ability of fungal isolate = clear zone diameter/ Fungal colony diameter: Biomass determination: Fungal cultures was filtered after incubation period and dried at70°C for 24 hours and weighed.

Cellulase assay: Enzyme activity was determined by using 3,5-Dinitrosalycilic acid (DNS) according to (20 , 19) by using two substrates CMC and filter paper, so we mentioned to the first CMCase method and to the second FPase method.

Improving cellulase productivity of fungal isolate by mutation:

1- Mutation by UV light: The method of (14) was followed by exposure 5ml of spore suspension to UV light with 254nm for (0, 5, 10, 15, 20, 25, 30) min.

2- Mutation by Gamma irradiation: The spore suspension was exposed to Gamma irradiation with 2 doses, 2 and 4 KGy for 20 min. according to (5) method.

3- Mutation by Nitrosoguanidine (NTG): The mutation was done according to (39) method, by using 200mg /ml of NTG and the spore suspension was 1×10^7.

RESULTS AND DISCUSSION

Isolation and primary screening: Seven isolates have been achieved from soil the main natural store of microorganisms, Grouted on PDA many times to obtain pure cultures. A primary screening was done to find the cellulase productive isolate by using carboxy methyl cellulose agar as a carbon source, by determining the clear zone and growth zone after soaking plates in Red Congo dye.

The results in **Table (1) and Fig. (1)** showed that there are 3 isolates recognized as high cellulase production isolates which their pinky clear zone diameter were (12.74, 10.30, 9.48) mm for (T5, T1, T4) isolate respectively, so these isolate were selected, other isolates were produce cellulase in different degrees but less than above, this difference could be cause to the genetic difference between isolates, this result agree with some studies (9, 38). These results agree with (6) when he found two isolates of

Trichoderma of 23 fungal isolates from soil with high cellulase production. Tests done by (33) gave same results when cellulase production was detected of fungi and bacteria isolated from soil.

Table 1: Cellulase productivity of fungal isolates by clear zone detection

Isolate No.	Z/G
T1	10.30
T2	6.85
T3	8.64
T4	9.48
T5	12.75
T6	5.30
T7	7.28

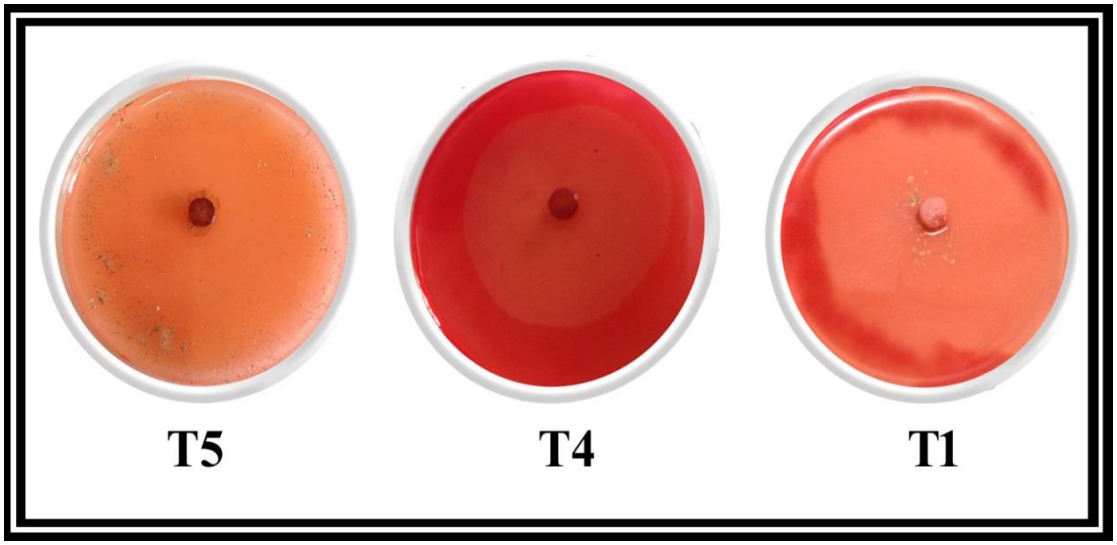

Fig.1: Primary screening of selected fungal isolates by clear zone detection

Secondary screening by using cellulase production media: Secondary screening for the isolates which showed the best efficiency in consuming cellulose in **table (1)** were done, they were three isolates T1, T4 and T5 respectively. The superiority of these isolates led to cultivate them on cellulase production media for 5 days to insure of their production activity.

There were clear differences between isolates in cellulase production by submerged culture as shown in **table (2),** the isolate T5 gave the highest cellulase productivity which was 14.75 U/ml by CMCase method and 1.28 U/ml by FPase method, followed by T1 isolate which was 9.53 U/ml by CMCase method and 0.16 U/ml by FPase method. The less productivity was 6.12 U/ml by CMCase and 0.03U/ml by FPase method for T4 isolate.

Table (2): Cellulase production by selected isolates (secondary screening)

Isolates	Cellulase production by CMCase method	Cellulase production by FPase method
T1	9.53	0.16
T4	6.12	0.03
T5	14.75	1.28

These results were close to (35) when they isolate Aspergillus and test their ability of cellulase production by solid state fermentation and there were many differences between isolates.

T. virens isolates from soil showed different amounts of cellulase by submerged culture (40). Other researchers mentioned that the difference between Trichoderma isolates in cellulase productivity could be because of the difference of media composition and circumstances conditions of production which induced productivity.

Selected isolate identification: According to primary and secondary screening results which showed the superiority of T5 isolate from soil, cultural and microscopic tests for identification, first it was grown on rose Bengal medium specialized for Trichoderma, and showed that the isolate was Trichoderma. According to classification keys in (4, 26) and consultation of Professor Hunsa Punnapayak and Professor Sehanat Prasongsuk from biomass unit/science College, they showed that the isolate is *Trichoderma reesei*. According to previous results this isolate was selected to conduct genetic improvement by different mutation methods.

Improving cellulase production by UV light in different periods: The selected isolate was exposure to UV light, and achieve mutant isolates according to mutation frequency as shown in table (3) which showed UV light exposure periods and the mutant frequency percentage. Three isolates for each treatment were selected and assigned depending on UV exposure period which showed ability to grow and cellulases production. The results of UV exposure showed that there is gradual increasing in cellulase activity by increasing UV exposure period as shown on table (4), the isolate Tr-UV 20 gave the highest cellulase productivity 17.38 U/ml by CMCase method in comparison with parent isolate 9.36 U/ml, and there is decline in enzyme productivity when the exposure period increased, the lowest production was 8.46 U/ml for Tr-UV 30 isolate by CMCase method. The biomass production is increased by increasing UV exposure period until the period which showed the highest cellulase production 24.67 gm/l. many studies proved the effect of UV light to detect mutants with high cellulase activity (13). Radiation is one of the most physical agents used in mutation especially UV light which gave a radiation about 260 nm for its killing properties and distinguish nitrogen bases. These results were similar to (7) when exposure *T. reesei* to UV light and achieve a mutant *T.reesei* RUT UV-15 which cellulase production was increased 22% in comparison with parent isolate. And similar to what found by (36) who exposure *T. reesei* YC-108 to UV light to improve cellulase production. Some researchers pointed to the ability to achieve many mutant isolates of *T. viride* (3) when exposed to UV light for 25 min., cellulase amount was (1461) U/gm in comparison with parent isolate (1350) U/gm. Our results were near to (31) when exposure *T.viride* to

UV light where the period 25 min gave the highest amount of cellulase (87)U/ml in comparison with parent isolate (53)U/ml. We could explain the effect of UV light in induced mutants by uniting between nitrogen bases especially between neighboring thymine - thymine on two DNA strips (32).

Table (3): Periods of UV light exposure for *T. reesei* isolate and mutant frequency%

Exposure period	Tr-UV-5 5min.	Tr-UV-10 10min.	Tr-UV-15 15min.	Tr-UV-20 20min.	Tr-UV-25 25min.	Tr-UV-30 30min.
Mutant frequency (%)	7.52×10^{-5}	8.55×10^{-5}	6.94×10^{-5}	7.66×10^{-5}	5.33×10^{-5}	7.20×10^{-5}

Table (4): efficiency of *T. reesei* isolate exposed to UV light for different periods in cellulase production

UVlight Exposure period	Biomass gm/L.	Cellulase production U/ml ByCMCase method	Cellulase production U/ml By FPase method
0	7.58	9.36	1.50
5	10.44	10.25	1.66
10	12.60	10.89	1.75
15	17.53	13.82	2.25
20	24.67	17.38	4.55
25	20.58	11.51	1.34
30	16.99	8.46	1.05

Improving cellulase production by Gamma radiations in different periods: Gamma radiation was used for increasing cellulase production by using two doses (2, 4) KGy under the same conditions used in previous test, three isolates were selected and assigned depending on radiation doses which showed ability analyze cellulose by determine clear zone which was (13.53)mm for Tr2-G2 isolate. Results on table (5) showed that cellulase activity was highest for Tr2-G2 isolate which was (28.55) U/ml by CMCase method and (7.49) U/ml by FPase method. The activity was decreased when the dose was 4KGy for the same isolate and the activity became (20.43) U/ml by CMCase method and (5.60) U/ml by FPase method, while the lowest cellulase activity for Tr3-G4 isolate was (10.55) U/ml by CMCase method and (1.85) U/ml by FPase method, in spite of that it was higher than parent isolate which was (9.58) U/ml by CMCase method and (1.55) U/ml by FPase method. The biomass was high (31.76) gm/L for Tr2-G2 isolate in comparison with parent isolate (7.90) gm/L. This increasing in cellulase activity could be refer to the activated effectiveness of radiation on some cell metabolic pathways which cause to induce improved mutants of *T.ressei* which possess positive effectiveness on clear growing and cellulase

production. These results were similar to (32) who achieved to improve cellulase production by *Trichoderma reesei* by using many doses of Gamma radiation, who gained many mutant isolates that more cellulase reproduction than parent, Tr-M21 isolate was the highest reproduction (9.09) U/ml at 250 Gy. Other researchers pointed that the dose 0.5KGy gave more cellulase activity for *Chaetomium Cellulyticum* NRRL 18756 by solid state fermentation (5), it was four times of parent activity by CMCase method. others said that *A. spp.* exposes to different doses of Gamma radiation (0-6) KGy (1), the dose o.5 KG gave the highest cellulase activity in comparison with parent which was (372) U/ml by CMCase method and (64) U/ml by FPase method, while it was (305) U/ml by CMCase method and (48) U/ml by FPase method for parent isolate.

Table (5): Efficiency of *T. reesei* exposed to Gamma Radiation with different doses on cellulase production

Mutants	Gamma radiation Doses	Biomass g/L.	Cellulase production U/ml By CMCase	Cellulase production U/ml By FPase
Tr1.G2	2KG	7.90	15.92	3.88
Tr1.G4	4KG	13.62	18.73	4.61
Tr2.G2	2KG	31.76	28.55	7.49
Tr2.G4	4KG	19.47	20.43	5.60
Tr3.G2	2KG	15.10	13.76	2.15
Tr3.G4	4KG	12.98	10.55	1.85

Improving cellulase production by Nitrosoguanidine (NTG): The mutation by Nitrosoguanidine was done according to (39). The clear zone were increased clearly to the three isolates which differentiate in their cellulase production ability, the highest clear zone was for Tr.NTG3 (15.27). As shown in table (6) the highest cellulase production was for Tr.NTG3 isolate (58.35) U/ml by CMCase method and (16.54) U/ml by FPase method, and that was emphasize results of clear zone. Second isolate in cellulase production was Tr.NTG2 (44.26) U/ml by CMCase method and (13.29) U/ml by FPase method, while the production was decreased for Tr.NTG1 (23.57) U/ml by CMCase method and (11.07) U/ml by FPase method.

On other hand Biomass was (38.22) gm/L. for Tr.NTG3 isolate and (25.95) and (15.68) gm/L. for Tr.NTG2 and Tr.NTG1, respectively. From results above it is clear that using NTG led to increase cellulase production five times in comparison with parent isolate (8.95) U/ml by CMCase method and (1.33) U/ml by FPase method. These results mentioned to the efficiency of using NTG which is one of strong mutagen which effect isolates and change their genetic structure, its low molecular weight 147 KD may be help to penetrate inside cells. These results were similar to (23) who mentioned to NTG as a chemical mutagen which led to improve cellulase production from *Trichoderma atroviride*. Moreover this result was identical to (12) by using *Trichoderma atroviride* isolate and treated with chemical mutagen which led to increase cellulase production.

Table (6): Efficiency of *T. reesei* exposed to NTG on cellulase production

Mutants	Biomass g/L.	Cellulase production U/ml By CMCase	Cellulase production U/ml By FPase
0	7.46	8.95	1.33
Tr.NTG1	15.68	32.57	11.07
Tr.NTG2	25.95	44.26	13.29
Tr.NTG3	38.22	58.35	16.54

CONCLUSION

Seven cellulytic fungal belong to Trichoderma species isolated from different soils of ancient town Ayutthaya in Bangkok/ Thailand. Primary and secondary screening methods have been used for the most cellulases productivity. Secondary screening performed by using cellulase production media.We have determined the improving cellulase productivity of fungal isolate by using three mutation by UV light, Gamma irradiation and Nitrosoguanidine (NTG). The activity was decreased when the dose was 4KGy for the same isolate and the activity became (20.43) U/ml by CMCase method and (5.60) U/ml by FPase method, while the lowest cellulase activity for Tr3-G4 isolate was (10.55) U/ml by CMCase method and (1.85) U/ml by FPase method.

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