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Research Article
Optimization of Growth Conditions for the Maximum Production of Secondary Metabolites from *Trichoderma harzianum* and their Biological Activities

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Abstract

Background and Objective: Fungal-strains are well familiar to produce potent therapeutic-moieties now days. *Trichoderma harzianum* is previously used as fungicide and have good applications in the treatment of soil for suppression of pathological microbial-strains. The current study was aimed to produce useful secondary metabolites in maximum quantity by optimizing the required conditions and to evaluate their biological activities. **Materials and Methods:** The experimental conditions were optimized by using various media (in various volumes), altering the incubation period and temperature, applying uniform and altering pH. Metabolites obtained were investigated for antibacterial, antifungal, cytotoxic, leishmanicidal, phytotoxic, insecticidal, α-chymotrypsin inhibitory and urease inhibitory potential. **Results:** Results revealed that the growth of *T. harzianum* was best supported by "Czapek Yeast Broth" at pH 7 with a maximum production of 1840 mg/100 mL followed by Sabouraud Dextrose Broth at pH 7 with 1580 mg/100 mL production with 15 days incubation at 30°C. The *T. harzianum* showed a significant antibacterial effect (inhibition %) against *S. dysentery* (96), *S. aureus* (94), *S. typhi* (81), *E. faecalis* (80), *A. parasiticus* (96), *A. flavus* (61) and *A. niger* (50). No leishmanicidal activity was observed in all tested samples. A moderate (65%) phytotoxic and mild (14.6%) insecticidal activity was observed. In enzyme inhibitory assays mild α-chymotrypsin (15.1%) and moderate urease (41.1%) inhibition was observed. **Conclusion:** The maximum secondary-metabolites of *T. harzianum* can be obtained by utilizing the above mentioned experimental conditions; that can be best therapeutic alternatives for the treatment of various pathological diseases.

Key words: *Trichoderma harzianium*, Czapek Yeast Broth, secondary metabolites, *in vitro* biological screening

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In biological research "fungi" have a very significant place, not only because of its widespread distribution but also being a source of production of very effective remedies. The term "metabolite" is strictly implicated for small molecules that are produced during metabolism by any organism, being classified into either primary metabolites and/or secondary metabolite. The primary metabolites are directly utilized by that organism for normal body functions such as growth, development and reproduction, while secondary metabolites are distinguished compounds that have an ecological importance. Secondary metabolites are involved in communication, cure and attraction/repulsion of other organisms[1].

Many micro-organisms including fungi are used as an important tool in biological research, being used by geneticists, biochemists, cytologist, mycologists and microbiologists as a model and as a bio-transformer etc. A number of fungal strains have been used for the production of secondary metabolites and those metabolites have proved their importance in medical and industrial uses. Among those valuable fungal strains one is the *Trichoderma,* which is most commonly found in agricultural land. Species of this genus i.e., *Trichoderma harzianum*, *Trichoderma hamatum* and *Trichoderma viride* are well known for its uses as bio-control agents in controlling plant fungal pathogens[2]. Among the members of *Trichoderma*, *Trichoderma harzianum*, which reproduces asexually with branched conidiophores have proved to exhibit very potent antifungal activity against *Macrophomina phaseolin* and *Fusarium solani*[3]. The *T. harzianum* cultured filtrates have been studied against *Meloidogyne javanica*'s eggs and are found very significant (90% inhibition)[4]. This species also showed a significant control on charcoal stem root rot disease of *Cucumis melo*[5].

The current study was aimed to produce the maximum secondary metabolites of *T. harzianum* by optimizing the experimental conditions, such as temperature, incubation period, medium (selection) and pH. The obtained secondary metabolites were then screened for various biological activities.

MATERIALS AND METHODS

Location of the study: The secondary metabolites production, extraction, fractionation and antimicrobial activities were carried out in the research laboratories of Centre of Biotechnology and Microbiology, University of Peshawar, conducted at HEJ Research Institute of Chemistry, International Centre for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan.

Chemicals, reagents, solvents and drugs: Czapek Yeast Agar (CYA) used was purchased from High media laboratories USA, Czapek Yeast Broth (CYB), Sabouraud Dextrose Broth (SDB), Potato Dextrose Broth (PDB), Bannerot Synthetic Medium (BSB), glycerol, Etoposide, Ampicilin, Miconazole, E-medium, phenol reagent, alkali reagents, sodium nitroprusside, N-Succinyl-Phenylalanine-p-Nitroanilide (NSPPN), Thiourea, fetal bovine serum (FBS) and Roswell Park Memorial Institute medium (RPMI-1640) were purchased from Sigma Aldrich, USA. Solvents (commercial grade) for extraction and fractionation were purchased from Burhani enterprises, Pakistan, while DMSO (analytical grade) was purchased from Merck, Germany.

Sampling, duration, isolation, purification and preservation of spores: The 50 g soil sample was collected from Malakand agency, Pakistan, from root soil of *Psidium guajava* in April, 2014. The study was conducted in a period of 16 months i.e., April, 2016 to August, 2017. The collected samples were serially diluted and inoculated in sterile petri-plates using CYA as medium and incubated at 25°C. The mix growth, so obtained was sub-cultured to get pure *T. harzianum* spores. For isolation of pure spores 1% detergent solution (5 mL) was used. The purified spores were preserved at -20°C in 50% glycerol-spore suspension (50:50 v/v).

Optimization of growth conditions: Four different types of fungal media including CYB, SDB, PDB and BSB were compared under different conditions for maximum biomass and secondary metabolites production. The CYB medium was selected, autoclaved and inoculated with 1 mL mycelia stock solution, incubated at temperature of 25°C in a shaking Incubator. After 16 days of incubation, the pH was determined and filtration was done, the filtrates were treated with concentrated hydrochloric acid followed by chloroform ($CHCl_3$). The above mixture was then allowed to settle for few minutes, the lower layer ($CHCl_3$ layer) was separated. The $CHCl_3$ was evaporated under reduced pressure using rotary evaporator and the semi-solid crude extract was collected. The above mentioned protocol was implicated for all the media at different temperatures i.e., 25, 30 and 35°C, respectively to find out the best supporting media and ideal temperature for the maximum production of secondary metabolites. The optimum incubation duration

was also determined in same manner following the above protocols for days i.e., 1, 3, 6, 9, 12, 15 and 18 days, respectively[6].

For determination of the optimized pH of the media, sterilized flasks (100 mL) were taken in which 9 flasks served for each media. The pH of each flask was adjusted as 3, 4, 5, 6, 7, 8, 9, 10 and 11. Each flask containing the above broth media was inoculated with 1 mL stock mycelial solution and was incubated at 25°C with continuous shaking. Secondary metabolites were obtained as described earlier and the best pH was selected (based on the maximum production of secondary metabolites). The same protocol was followed for the determination of suitable volume of the media. Different volumes of the media used[7] were 30, 40, 50, 60, 70, 80, 90 and 100 mL.

Fractionation: The crude extract of *T. harzianum* (CETH) was mixed with equal volume of water and soaked overnight. That solution was then extracted with various organic solvents in ascending order of polarity i.e., n-hexane (3×0.5 L), $CHCl_3$ (3×0.5 L) and etylacetate (3×0.5 L) to get the maximum soluble fractions of the respective solvents[8].

Antibacterial activity: The CETH was screened for antibacterial activity against *Enterococcus feacalis* (ATCC 14506), *Staphylococcus aureus* (ATCC 43300, Pakistan), *Pseudomonas aeruginosa* (ATCC 27853, Abbott, Pakistan), *Escherichia coli* (ATCC baa 2452, Pakistan), *Shigella dysentery* (ATCC 13313, England) and *Salmonella typhi* YS 1646 (ATCC 202165, Pakistan) using previously reported protocol of Ullah *et al.*[9].

Antifungal activity: The CETH was screened for antifungal activity against *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus paraciticus*. Previously reported method of Lavermicocca *et al.*[10] was implicated for the antifungal assay.

Leishmanicidal activity: The previously published protocol of Gardlo *et al.*[11] was executed to the determine the Leishmanicidal activity (against *Leishmania major*) of CETH and various fractions.

Brine shrimp bioassay (cytotoxic activity): To determine the *in vitro* cytotoxic activity of CETH and subsequent fractions, the protocol of Habiba *et al.*[12] was used with slight modification. The modification was doses used such as 10, 100 and 1000 µL (stock solution 20 mg mL^{-1} in methanol).

Phytotoxic activity: The phytotoxic activity against *Lemna minor* of the CETH and various fractions were determined, using previously published protocol of Ahmad *et al.*[13].

Insecticidal activity: The contact insecticidal potential of CETH and fractions was determined against *Rhyzopertha dominica*, *Tribolium castaneum* and *Callosobruchus analis*. The previously published protocol of Pavela *et al.*[14] was implicated for this activity.

α-Chymotrypsin inhibition: The CETH and fractions were screened for α-chymotrypsin inhibitory potential using previously published protocol of Marasini *et al.*[15].

Urease inhibitory activity: The previously published protocol of Ullah *et al.*[16] was executed to evaluated the urease inhibitory potential of CETH, n-hexane and ethyl acetate fraction.

Statistical analysis: Statistical analysis was performed using the desired database for individual activity, such as Perrella Scientific, Fenny Analyzer GraphPad Prism 5 statistical package and Microsoft excel. Results were presented in percentage, IC_{50}, LD_{50} and Mean±SEM.

RESULTS

Optimization of media and incubation time: Results for production of secondary metabolites of *T. harzianum* at different incubation time is shown in Fig. 1 and 2. Duration-wise the maximum production was observed in 9-18 days. The optimum incubation time for CYB was 15 days, as maximum quantity of secondary metabolites of 1750 mg/100 mL were produced. From the results it was also cleared that CYB was the best medium for mycelial growth and secondary metabolite production in with incubation time of 15 days.

Optimization of pH: The effect of pH on the production of secondary metabolites was presented in Fig. 3 and 4. It was obvious from the figures that maximum quantity of the secondary metabolites (14 mg/100 mL) and mycelial biomass (1500 mg/100 mL) was produced at pH 7 in the CYB media, while in other media the production of secondary metabolites was low at any pH, however, their maximum response were noticed at pH 7.

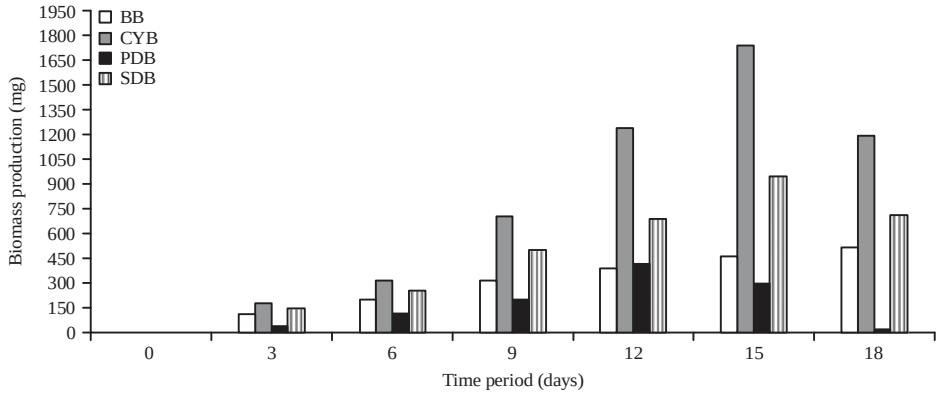

Fig. 1: Weight of mycelial biomass productions at various time periods and media

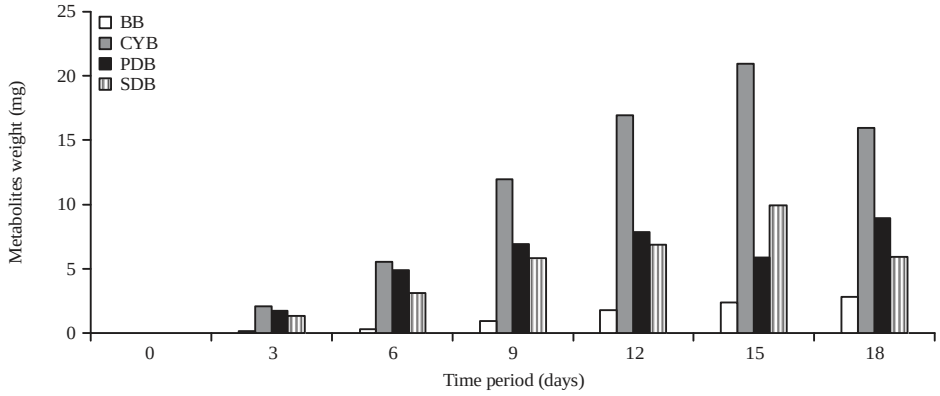

Fig. 2: Weight of secondary metabolites productions at various time periods and media

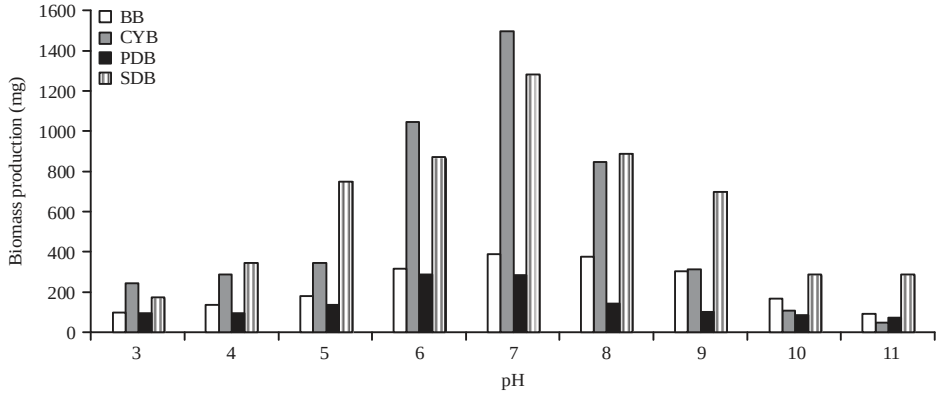

Fig. 3: Mycelial biomass productions at various ranges of pH in different media

Optimization of volume: The results of optimization experiments for volume of the media were presented in Fig. 5 and 6. Upon raising the volume of the CYB culture medium to 100 mL, the fungus produced maximum quantity of secondary metabolites (14.6 mg) and mycelial biomass (1890 mg). Other media also showed a maximum response on 100 mL, in contrast to CYB, the production of secondary metabolites was low in other tested media.

Optimization of temperature: The effect of temperature was presented in Fig. 7 and 8. It was evident from the results that maximum production of biomass was observed at 30°C for all the media used. However, the results showed that the maximum amount of secondary metabolites (23.7 mg/100 mL) and mycelial biomass (1840 mg/100 mL) were produced in CYB at same experimental conditions.

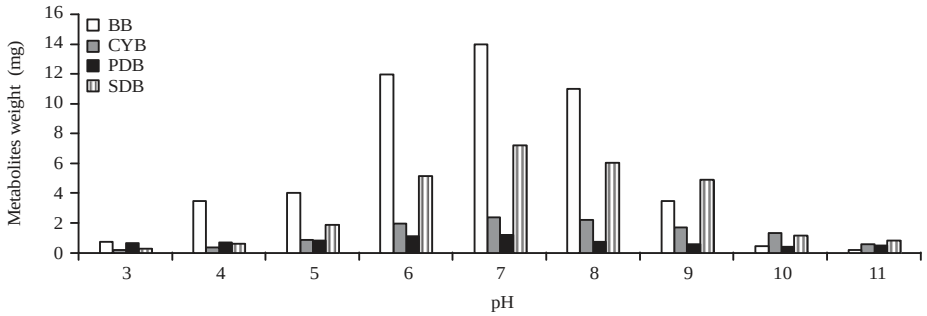

Fig. 4: Metabolites production at various ranges of pH in different media

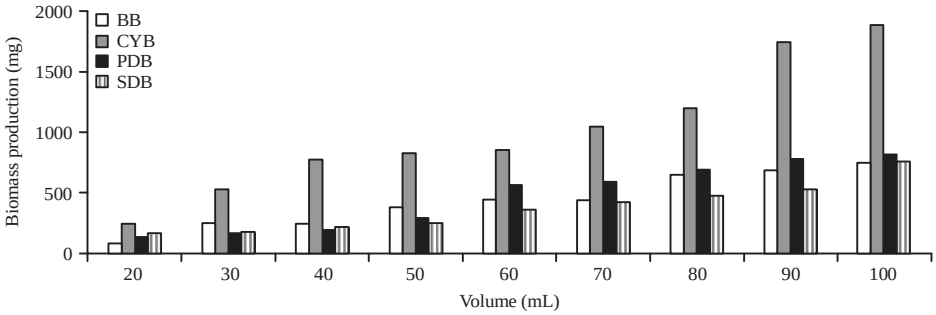

Fig. 5: Mycelial biomass productions in various ranges of volume in different media

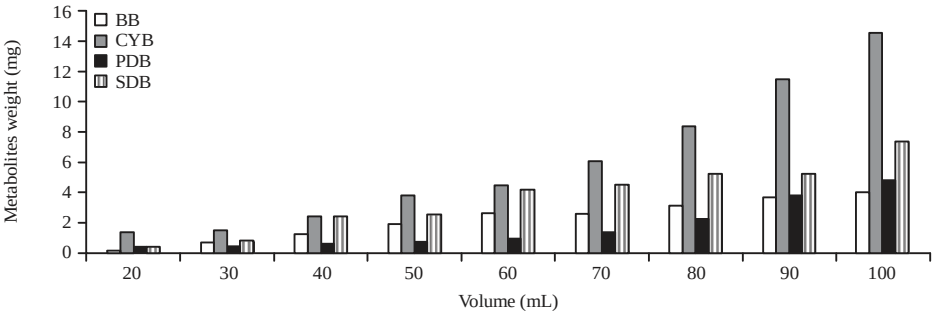

Fig. 6: Metabolites production in various ranges of volume in different media

Antibacterial activity: The results of antibacterial activity were shown in Fig. 9. Significant activity was shown by the CETH at 30 µL against *S. dysentery* (96), *S. aureus* (94), *S. typhi* (81) and *E. faecalis* (80), moderate against *E. coli* (48) and low against *P. aeruginosa* (36).

Antifungal activity: The results of antifungal activity were presented in Fig. 10. The results showed that at 10 µL, significant activity was observed for *A. parasiticus* (95%) and good against *A. flavus* (79%) and *A. niger* (66%). At 20 and 30 µL, the percent zone of inhibition was: *A. parasiticus* (95. 96), *A. flavus* (62. 61) and *A. niger* (57. 50).

Leishmanicidal activity: The results revealed that the tested samples didn't show any significant leishmanicidal activity. Their IC_{50} were more than 100 and no appreciable inhibition was observed.

Phytotoxic activity: The results of phytotoxic potential of CETH and various fractions were shown in Fig. 11. A maximum phytotoxic effect was observed in case of ethylacetate fraction (65%) followed by CETH (20%) and n-hexane fraction (30%) at 1000 µg mL^{-1}, respectively. At lower doses no net significant effect was noticed.

Insecticidal activity: Results of insecticidal activity were presented in Fig. 12. The maximum insecticidal effect (35%) was observed in case of ethylacetate against *T. castaneum* and *R. dominica,* followed by n-hexane and CETH (30%) for *R. dominica*.

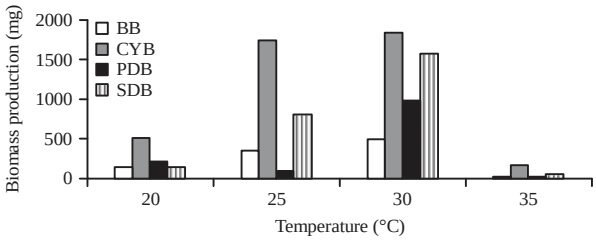

Fig. 7: Biomass productions in various ranges of temperature in different media

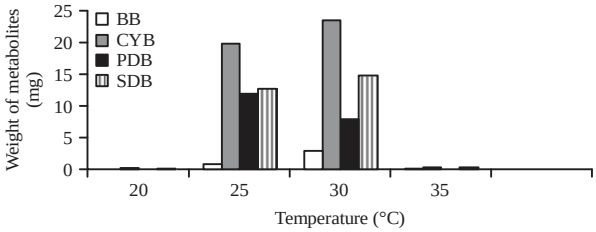

Fig. 8: Metabolites production in various ranges of temperature in different media

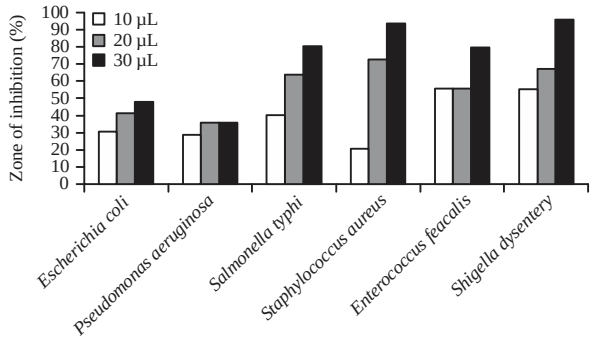

Fig. 9: Inhibition (%) by CETH against the selected bacterial species

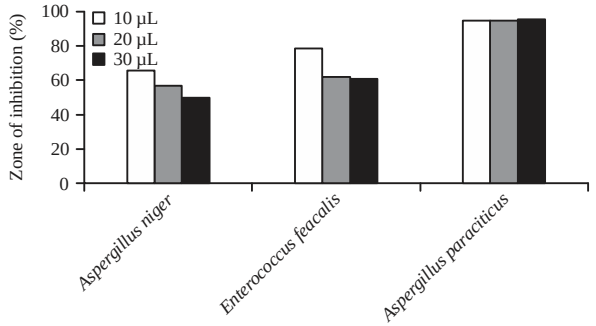

Fig. 10: Inhibition (%) of CETH against test fungal species

Brine shrimp bioassay (Cytotoxic activity): The results of cytotoxic activity were shown in Fig. 13. The CETH showed moderate activity (46.6%) at 1000 µg mL^{-1} followed by ethylacetate (50%) and n-hexane fraction (43.3%) at 1000 µg mL^{-1}, respectively.

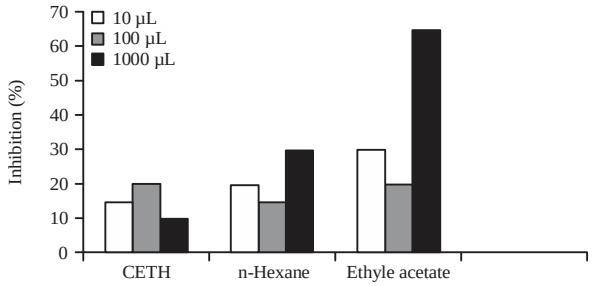

Fig. 11: Phytotoxic activity of crude extract and various fractions at different concentrations

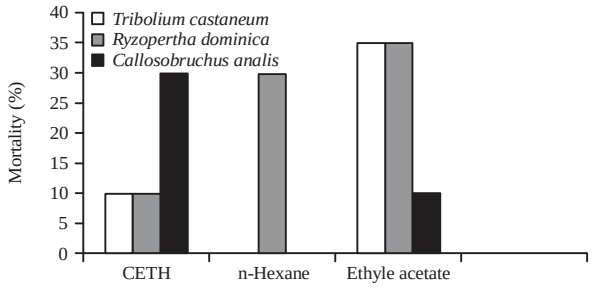

Fig. 12: Insecticidal activity of CETH and fractions

Urease inhibitory assay: The results of urease inhibitory potential were shown in Fig. 14. A maximum effect was observed in case of ethylacetate fraction (41.4%) followed by CETH (14.6%), similarly the n-hexane fraction showed very weak 5.2% urease inhibitory potential.

α-chymotrypsin inhibitory assay: The results of α-Chymotrypsin inhibition assay were shown in Fig. 15. Among the tested samples a maximum effect was observed in case of n-hexane fraction 17.1% followed by ethylacetate fraction and CETH i.e., 15.1 and 1.4%.

DISCUSSION

It is highly desired to discover excellent remedies for diseases that are more potent, comparatively less toxic (having low or no adverse effects) and effective in treating resistant pathological strains. Research on medicinal plants and naturally (readily available active moities) available therapeutics was a good era but in the current scenario, researchers are highly impressed to work on biotransformed

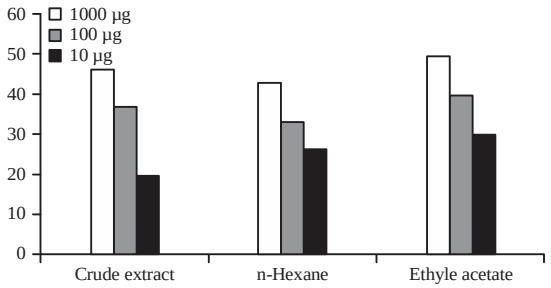

Fig. 13: Inhibition (%) of CETH and fractions against Brine shrimp

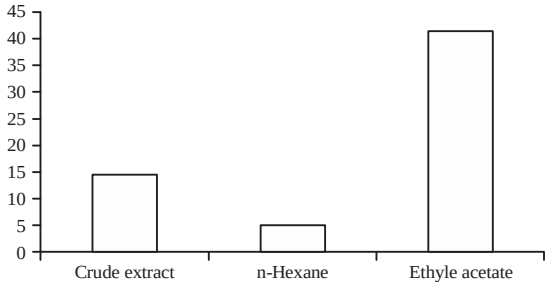

Fig. 14: Inhibition (%) of CETH and fractions against Urease

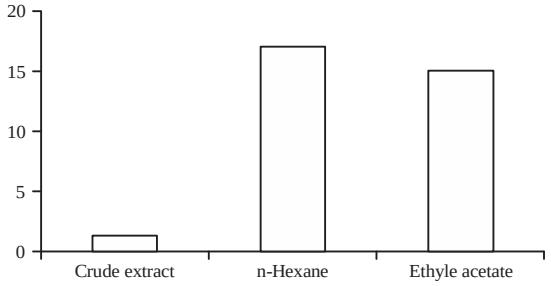

Fig. 15: Inhibition (%) of CETH and fractions against α-chymotrypsin

chemicals. Keeping in mind that; microbes, especially fungal strains have been used in the past to produce many drugs such as antibiotics, hormones or other biotechnological products; the fungal specie, *Trichoderma harzianum*, which has been used previously for controlling the plant pathogens, has been investigated for the production of some valuable agents (secondary metabolites). The experimental parameters were optimized such as the CYB is used as medium in 100 mL volume, while the pH was maintained at 7 and 30°C for 15 days; maximum biomass and secondary metabolites were obtained. These results agreed with those reported in the literature[17]. The isolated secondary metabolites when evaluated for its possible biological screening. The maximum antibacterial activity was observed in case of *S. dysentery* followed by *S. aureus*, *E. faecalis* and *E. coli*. Many familiar diseases have been caused by these pathogens especially *S. dysentery* is famous for a well known term "Shigellosis" that is typically comprised of the symptoms such as; severe acute gastro-enteritis, abdominal cramps, bleeding in stools etc. many drugs have been used for its management including nalidixic acid but unfortunately some plasmid-induced resistance have been developed against this antibiotic[18] the resistance has been observed in Africa, while in while its type 1 (the *Shiga bacillus*) have been notified in Bangladesh and Africa, some other cases have reported for the comparative studies among antibiotics including nalidixic acid and norfloxacin[19] while in another study the same type 1 was found resistant to norfloxacin, ciprofloxacin, ampicilin, cotrimoxazole and nalidixic acid[20]. Similarly, the *S. aureus* is also common pathogenic specie, which is a leading cause of diseases including pimples, scaled skin syndrome, abscess, folliculitis, boils and impetigo (skin infections). It can also cause some life-threatening events such as osteomyelitis, meningitis, pneumonia, bactermia, sepsis and even shock (toxic shock syndrome)[21-23]. In many cases this specie has been found resistant to various drugs including advance vancomycin which is supposed to be drug of very broad spectrum and high potency against those pathogens which are resistant to a number of anti-bacterial agents[23]. In previous reports, it has also mentioned that the *S. aurous* cause production of Panton-Valentine leukocidin, a cytokine that causes the destruction of leukocytes and necrosis of tissues; and this mediator have been reported to cause necrotic hemorrhagic pneumonia in 85% of cases[24]. Similarly the *E. faecalis* is the group D pathogen, being a leading cause of nosocomial infection, among which UTI is on the most common one. The infections caused by this organism is raising day by day, the reason is same to the above mentioned, i.e., the resistance. Even this organism has been reported to be resistant to a number of antibiotics including the vancomycin[25]. Similarly the next pathogen to human is fungi the most common among which were *Aspergillus paraciticus* which is among fungi producing mycotoxin and aflotoxin the most naturally occurring cancer causing agents[26-28]. Similarly *Aspergillu flavus* a cause of invasive aspergillosis in human and also infect many crops is yet another well known pathogen that not only effect human life directly but indirectly effect the economy. It also being another source of production of aflatoxin, human allergen and mycotoxin[29,30]. It is specified in the production of aflatoxin B1 and G1, the aflatoxin B1 is highly potent cancer causing agent with TD

0.0032 mg kg^{-1}/day in rats [31]. Similarly *Aspergillus niger* being the most common in *Aspergillus* genus was typically the infectious agent of fruits and vegetable leading to black mould effect the economy[32].

The tested samples showed very good antimicrobial against the above pathogenic strains of bacteria and fungi. Leishmaniasis is the disorder associated with the bite of a sand fly and is most common in tropical region, its treatment is either highly expensive or quite toxic compounds are used to treat it[33]. Discovering effective phytotoxic agents; to kill the weeds and enhance the current crop growth in order to get maximum production is also very important aspect for research. It not only for single man advantage but also serve to support the net economy of the nation as well[34]. While the other aspect i.e., discovering insecticides for insects such a *Callosobruchus analis*, *Ryzopertha dominica* and *Tribolium castaneum* is also an interesting field. The currently available insecticides were typical organo-phosphorus poisons, are highly toxic, not only for human (contact) but their drains can also effect the river and so on sea environment[35]. The other interesting area for research was to discover inhibitors for enzymes which helps in the regulation of normal life including urease and chymotrypsin, the urease are important in many aspects such as to control the growth of *H. pylori*, not only at gastric level but renal as well. Controlling urease and chymotrypsin both at the same time by a single moiety will be highly effective in treating gastro-renal disorders[16]. The secondary metabolites of *T. harzianum* were found very effective in the control of *S. dysentery*, *S. aureus*, *E. faecalis* and *E. coli*, while also exhibited very good antifungal activity against the test fungal strains. Rest of the screenings performed very of mild to moderate effectiveness. The production of maximum secondary metabolites in order to decrease the cost of production, duration of incubation etc. directly decreases the cost of treating such diseases.

This study contains data regarding the production of potent antimicrobial agents in bulk, which could help in combating human pathogenic diseases; two aspects are highly appreciable, (1) Potent antimicrobial activities could open gates for the further studies to isolate the potent drugs that could help in managing currently resistant pathogenic strains and (2) Maximum production of secondary metabolites in single run will decrease the cost of treatment; which is really a load on low economy developing countries.

CONCLUSION

It can be concluded that the maximum "secondary metabolites" from the *Trichoderma harzianum* can be obtained using Czapek Yeast Broth as medium at 30°C and pH 7 with the incubation period of 15days.

SIGNIFICANCE STATEMENT

The secondary metabolites produced by *Trichoderma harzianum* proved to exhibit potent antimicrobial effect against *S. dysentery*, *S. aureus*, *E. faecalis* and *Aspergillus* species; hence these secondary metabolites can be used for the treatment of diseases caused by above mentioned species, after validation through clinical studies.

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