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Screening and Media Optimization for Enhancing L-asparaginase Production, an Anticancer Agent, from Different Filamentous Fungi in Solid State Fermentation

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of present study was to screen new potent fungal isolates and microorganisms possessing extracellular L-asparaginase production capacity. In addition, optimization of cultural and environmental conditions required for enzyme production will be carried out for the highest L-asparaginase producer in solid state fermentation (SSF) technique using agro-industrial residues.
Study Design: Screening and physiological studies on the formation of L-asparaginase by *Trichoderma viride* F2 in order to obtain the optimum cultural and environmental conditions required for enzyme production.
Place and Duration of Study: Department of Microbial Chemistry, Genetic Engineering and Biotechnology Division, National Research Centre (NRC), Cairo, Egypt, between July 2013 and June 2015.
Methodology: Optimization of physical and nutritional parameters for enzyme production was investigated. Various locally available agro-industrial residues have been screened individually or as mixtures for L-asparaginase production. The combination of Rice husk (RH) with wheat bran

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(WB) (3:2) proved to be an efficient mixture for enzyme production as it gave the highest enzyme activity (71.87±3.19 U/g-ds) when compared to individual RH (66.71±2.76 U/g-ds) or WB (62.28±2.13 U/g-ds) substrates.
Results: Maximal L-asparaginase production (113.43±5.11 U/g-ds) by *T. viride* F2 was obtained with moisture content of 75%, an inoculums size of 1 x 10^8 spores/ml and an initial medium pH of 5.0 when incubated at 28ºC for four days. Presence of Tween 20 enhanced enzyme production by 1.19 folds. Glucose (1.0%), Casein (1.5%) and $MgCl_2$ (0.05%) were found to be the best carbon, organic nitrogen and ion sources, respectively. Supplementation of the medium with $NaNO_3$ (0.15%) as an inorganic nitrogen source further increased L-asparaginase production. Under these optimized conditions, L-asparaginase production by *T. viride* F2 was maximum with a yield of 276.5±13.4 U/g-ds in SSF, which was more than 19-fold enhancement in enzyme activity as compared to that obtained in the basal medium (SmF) (14.23±0.87 U/ml).
Conclusion: The results suggest that choosing a suitable substrate coupled with optimization of different parameters can improves enzyme production markedly. Moreover, the production of L-asparaginase from a process based on RH and WB as substrates in SSF is economically attractive due to abundant substrates availability in agriculture-based countries with cheaper cost.

Keywords: L-asparaginase; filamentous fungi; optimization; screening; solid state fermentation.

1. INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) is known to be the most proper drug for the treatment of acute lymphoblastic leukemia (ALL) and other cancer diseases. It has been and is still one of the most widely studied therapeutic enzymes by researchers and scientists worldwide. The production of this enzyme has a broader prospectus in industrial area and even in pharmaceutical industries as the microbial production of L-asparaginase is inexpensive [1-4]. The main function of L-asparginase towards the treatment of cancer is to hydrolyze L-asparagine, an essential amino acid, to L-aspartic acid and ammonia, and to a lesser extent, the hydrolysis of L-glutamine to L-glutamate. Although different types of tumor cells require L-asparagine for protein synthesis, they are deprived of an essential growth factor in the presence of L-asparaginase, thus, resulting in cytotoxicity of leukaemic cells [5-6]. Furthermore, recent studies have reported potential application of L-asparaginase in prevention of acrylamide formation in fried potatoes and similar food products. In this connection, researchers reported that acrylamide formed from asparagines during the browning that occurs in baking, frying, and grilling of products made from potato or cereal at temperatures exceeding 120ºC. This substance may be carcinogenic and detrimental to human genes as it can cause cancer to many individuals [7-9].

Administration of L-asparaginase from bacterial origin can cause hypersensitivity in the long term used, leading to allergic reactions in the tissues of patients, resulting in anaphylactic shock and may cause neutralization of the drug effect. Therefore, the search for a new serologically different L-asparaginase with similar therapeutic role and less adverse effects is highly recommended. L-asparaginase occurs widely in nature and their presence has been reported in plants, certain animal tissues and microorganisms including bacteria, yeast and filamentous fungi [10]. However, microbial L-asparaginases are preferred because microorganisms produce abundant amounts of the desired product in a short period of time and can be easily manipulated through genetic engineering to generate more stable enzymes with altered properties than other sources [11]. Filamentous fungi are one of the most important asparaginase sources for industrial application because fungal enzymes are usually excreted extracellularly, facilitating extraction from the fermentation media with low cost and high productivity and are more resistant to harsh climatic conditions [12].

Optimization of nutritional and physical requirements of microorganism is important to develop and control the economic feasibility of any bio-process. The optimum levels of process parameters for maximum enzyme production are unique for each microorganism. In this concern, no defined medium has been established for the optimum production of L-asparaginase from different microbial sources. Therefore, the aim of present study was to screen new potent fungal isolates and microorganisms possessing extracellular L-asparaginase production capacity. In addition, optimization of cultural and environmental conditions required for enzyme production will be carried out for the highest

L-asparaginase producer in solid state fermentation (SSF) technique using agro-industrial residues.

2. MATERIALS AND METHODS

2.1 Microorganisms

Different fungal cultures were locally isolated from samples of soil collected from Giza, Egypt, by employing the dilution plate method according to Palaniswamy et al. [13], whereas others obtained from the culture collection of Microbial Chemistry Department, National Research Centre (NRC).

2.2 Substrates

Various agro-industrial substrates (maize, rice bran, rice husk, wheat bran, wheat germ, rice straw, cotton seed wastes) were collected from the local fields, Kalubia governorate, Egypt, washed with tap water, air-dried at dry season for 15 days, and finally packed and stored in plastic bags at room temperature for later use.

2.3 Pre-treatment of Rice Straw

Rice straws (RS) were collected from the local rice fields, Kalubia governorate, Egypt. The air-dried straws were cut into one cm, dried at 80ºC for 24 h in air-circulation oven, then ground to uniform size (6 meshes) in an electric grinder, finally packed and stored in plastic bags at room temperature for use. Rice straw (5%) in 160 ml of 1% NaOH aqueous solution was pressure cooked at 121ºC for 1 h. Treated rice straw were then collected by filtration and extensively washed with distilled water. The pH was adjusted to 5.5 with 1N HCl and dried overnight at 45ºC in a forced-draft oven.

2.4 Qualitative Screening of L-asparaginase Producing Fungi

The isolated filamentous fungi and those from our culture collection were subjected to rapid screening for L-asparaginase production by agar plate assay as reported by Gulati et al. [14]. The fungal strains that showed pink zone around the colonies indicated L-asparaginase production and were selected for quantitative enzyme assay.

2.5 L-Asparaginase Production by Solid State Fermentation

Enzyme production was carried out in 250 ml flasks containing 5g RS moistened with 0.01 M Phosphate buffer pH 6.0 to a moisture level of 66%. All flasks were sterilized at 121ºC for 20 min. Two ml aliquots from each spore suspension (1×10^6 spores/ml) were used to inoculate the flasks and then incubated at 28ºC for four days.

2.6 Enzyme Extraction

The crude enzyme was extracted from the fermented substrate by adding 50 ml of 0.05 M Tris-HCl buffer (pH 8.0). The flasks were kept on a rotary shaker (150 rpm) at room temperature for 30 min. The slurry obtained was centrifuged at 5000 rpm for 10 min at 4ºC. Finally, the clear supernatant was collected and used for extracellular L-asparaginase assay.

2.7 L-Asparaginase Assay

L-Asparaginase activity was determined by estimating the amount of ammonia liberated from L-asparagine following the method presented by Imada et al. [15]. One unit (U) of L-asparaginase was defined as the amount of enzyme that liberates 1 µmole of ammonia under optimal assay conditions. The enzyme yield was expressed as a unit per gram dry substrate (U/g-ds). Protein content in the crude enzyme preparation was determined according to Bradford [16].

2.8 Optimization of L-asparaginase Production

The parameters studied included screening of different solid agricultural substrates (pretreated rice straw, rice bran, rice husk, wheat bran, wheat germ, cotton seed and maize), initial medium pH value (3.0-8.0 adjusted with 1N HCl or 1N NaOH), initial moisture content (within the range of 50 to 86%), duration (during the fermentation, the flasks were taken at regular intervals of 24 hrs), incubation temperature (25, 28, 35 and 40ºC). The influence of inoculum size (1×10^4 - 1×10^9) and surfactants (Tween 20, Tween 60, Tween 80 and Triton X-100 at 0.1% w/v) were determined. The effect of carbon source (Glucose, sucrose, maltose, fructose, xylose, galactose, arabinose, soluble starch and raffinose at 1.0% w/v), various organic (Urea, yeast extract, casein, malt extract, proline and peptone at 0.5% w/v) and inorganic nitrogen sources (ammonium sulphate, ammonium nitrate, ammonium phosphate, sodium nitrate and ammonium chloride at 0.1% w/v) on L-asparaginase production was also evaluated. Effect of inducers (L-asparagine, L-glutamine,

L-aspartic acid and L-glutamic acid at 0.1% w/v) and metal salts ($MgCl_2$, $AgNO_3$, $HgCl_2$, and $FeCl_3$, $ZnSO_4$, $CdCl_2$, KCl, $MnCl_2$, $FeCl_3$, $CoCl_2$, KH_2PO_4, NaCl and $BaCl_2$ 0.01% w/v) on enzyme formation was investigated. All the experiments were conducted in triplicate and the data were expressed as mean±standard deviation [17].

3. RESULTS AND DISCUSSION

3.1 Screening of Different Filamentous Fungi and Isolates for L-asparaginase Production

3.1.1 Qualitative analysis

In the rapid qualitative plate assay method, out of fifty two fungal species and isolates screened for L-asparaginase activity, 47 fungal sp. and isolates gave positive test with variable degrees depending upon the intensity of the produced pink color (Fig. 1). The formation of pink color can be interpreted by the breakdown of amide bonds in L-asparagine by L-asparaginase with accumulation of ammonia in the medium. *Trichoderma viride* F2, *Penicillium politans* NRC 510, *P. purpurescens* and *Aspergillus terreus* NRRL 265 exhibited deep pink color. While *Aspergillus phoenicis*, isolate DPG 11 and isolate FDH 115 gave negative agar plate test. In this concern, many investigators reported the validity of the previously mentioned method for the primary screening of L-asparaginase from *Aspergillus* and *Penicillium* sp. [18-20]. For further confirmation, spectrophotometric quantitative method is required.

3.1.2 Quantitative analysis

The capability of different filamentous fungal strains and isolates on L-asparaginase production on rice straw is shown in Table 1, from which it is clear that mostly all selected fungi from the tested set produced extracellular L-asparaginase in different proportions. However, *Trichoderma viride* F2 gave the highest extracellular L-asparaginase production (58.4±2.52 U/g-ds) followed by *Penicillium javanicum* (47.9±2.13 U/g-ds), Isolate DH 314 (44.9±2.25 U/g-ds) and Isolate TH 13 (36. 6±1.24 U/g-ds). Therefore, *T. viride* F2 was chosen for further studies.

3.2 Comparative Evaluation of SmF and SSF System for Enzyme Production

L-asparaginase production from *T. viride* F2 in solid state fermentation (SSF) and submerged fermentation (SmF) (Czapek Dox's liquid medium) [21] were compared in terms of their extracellular enzyme production in U/g-ds and U/ml, respectively. Production of total L-asparaginase by SSF (57.11±1.89 U/g-ds) was 4-fold higher than that of SmF (14.23±0.87 U/ml) (data not shown). The mechanism of depressive effect in modified Czapek Dox's medium (MCD) broth is thought to result from the presence of glucose metabolic products. Studies suggest that in the case of asparaginase biosynthesis, the depressive effect of carbohydrates may be a function of their ability to lower the pH value of the fermentation media [22]. This suggests that there may be increased accumulation of intermediate metabolites between substrate and product formation in submerged fermentation. This is also probably due to the difference in the physiological state of the microorganism in solid-state and submerged fermentations. Therefore, having considered the means of reducing disposal problem, rice straw can be effectively utilized by the potential strain for production of enzyme which is medically commercially important.

3.3 Screening of Different Agro-industrial Substrates for Enzyme Production

Selection of appropriate solid substrate is a crucial step for SSF [23]. In the present study, the initial screening of various substrates indicated that all the substrates tested promoted enzyme production with *T. viride* F2, however, maximum L-asparaginase titer (66.71 U/g-ds) was achieved in a medium containing rice husk followed by wheat bran (60.28 U/g-ds), while least enzyme production of 39.18 U/g-ds was noticed with rice bran (Fig. 2). Hymavathi et al. [24] reported L-asparaginase production from *Bacillus circulans* MTCC 8752 under solid state fermentation using different agricultural waste materials like red gram husk, bengal gram husk, coconut, and groundnut cake. Also various substrates have been used based on need and availability for enzyme production by SSF. Some of the substrates like wheat bran, rice straw, soya meal, husk, rice bran, bagasse, sawdust, wheat straw have been utilized by many researchers [25-27]. However, wheat bran was considered as the universal substrate among various substrates because it acts as a complete nutritious feed for microorganisms having all the ingredients and remains loose even under moist conditions providing a large surface area [28]. Moreover, the biochemical composition of wheat bran indicates that it contains various soluble

sugars like glucose, xylose, arabinose, galactose, etc. which are helpful for the initiation of growth and replication of microorganisms. In addition, higher L-asparaginase production on wheat bran may be possibly due to low lignin content and more amount of protein as compared to other substrates [29].

3.4 Screening of Mixed Substrates for L-asparaginase Production

Low bed porosity and consequent poor circulation of air adversely affects oxygen transfer and cooling in solid-state fermentations. Porosity of a bed of fine particles can be increased by mixing in some coarser solids that do not pack together closely [30]. As previously mentioned, from the substrates screened, rice husk (RH) and wheat bran (WB) were found to be the best substrates for L-asparaginase production, for this reason they were mixed in different ratios and screened for maximum L-asparaginase production (data not shown). Results obtained indicated that, from the different ratios screened RH and WB in the ratio of 3.0:2.0 on dry weight basis obtained maximum L-asparaginase production (71.87±3.19 U/g-ds) than using RH (66.71±2.76 U/g-ds) and WB (62.28±2.13 U/g-ds) alone under the same experimental conditions.

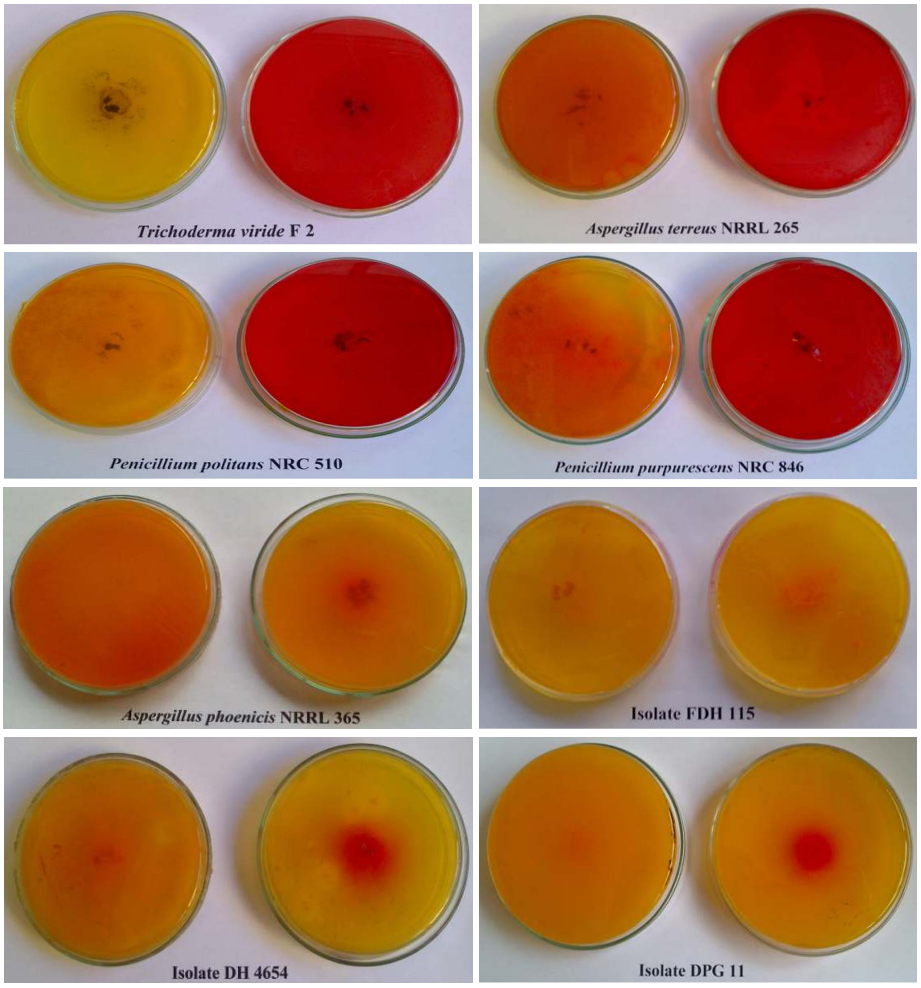

Fig. 1. Qualitative analysis for L-asparaginase production using the agar plate assay test
Left side = Control

Table 1. Quantitative analysis for extracellular L-asparaginase production from different filamentous fungi and isolates using rice straw as a sole carbon source for growth in SSF technique

No	Microorganism	mg protein / ml	L-asparaginase activity (U/g-ds)
1	*Aspergillus fumigatus* DSM 819	0.84	15.2±0.89
2	*Aspergillus niger* ASU 1	0.39	6.1±0.18
3	*Aspergillus oryzae* NRRL 3435	0.42	7.8±0.21
4	*Aspergillus oryzae* NRRL 447	0.61	15.1±0.72
5	*Aspergillus oryzae* NRRL 3484	0.49	1.9±0.16
6	*Aspergillus oryzae* NRRL 480	0.38	7.4±0.25
7	*Aspergillus oryzae* UEAC1	1.15	23.6±1.21
8	*Aspergillus oryzae* UEAR3	0.48	9.3±0.87
9	*Aspergillus oryzae* UNAC16	0.69	7.2±0.38
10	*Aspergillus oryzae* UNBC 7	0.45	21.4±2.14
11	*Aspergillus oryzae* UNBK 25	0.51	7.9±0.11
12	*Aspergillus phoenicis* NRRL 365	0.33	22.9±1.54
13	*Aspergillus terreus* NRRL 265	0.58	19.4±1.32
14	*Fusarium solani*	0.67	5.6±0.32
15	*Penicillium brevicompactum* NRC 829	0.44	6.2±0.12
16	*Penicillium funicolosum* NRC 258	1.53	5.8±0.16
17	*Penicillium javanicum*	0.64	47.9±2.13
18	*Penicillium politans* NRC 510	0.42	10.2±0.78
19	*Penicillium purpurescens* NRC 846	0.41	8.6±0.11
20	*Scopulariopsis brevicaulis* ASU 3	0.39	13.8±0.91
21	*Trichoderma* sp. 5	0.48	21.4±1.13
22	*Trichoderma* sp. 1	0.87	11.4±0.98
23	*Trichoderma viride*	0.92	18.1±1.24
24*	***Trichoderma viride* F2**	**0.48**	**58.4±2.52**
25	Isolate HAMZ 2	0.43	17.9±1.11
26	Isolate JANA 3	0.42	13.2±1.34
27	Isolate OMAR 1	0.51	24.8±1.14
28	Isolate HESH 97	0.49	6.4±0.56
29	Isolate DHI 7	0.47	10.1±0.73
30	Isolate SHI 7	0.76	16.3±1.16
31	Isolate AHI 7	0.33	8.9±0.87
32	Isolate FAZ 71	0.82	19.2±1.78
33	Isolate TH 13	0.56	36. 6±1.24
34	Isolate SOES 47	0.75	20.3±1.12
35	Isolate ALMO 93	0.47	25.3±1.62
36	Isolate FXBH 82	1.05	13.8±0.73
37	Isolate FASK 25	1.14	14.6±0.87
38	Isolate DH 417	0.25	5.3±0.24
39	Isolate HISB 75	0.39	21.6±1.61
40	Isolate DH 365	0.41	6.2±0.31
41	Isolate DH 4654	0.42	12.3±0.68
42	Isolate DH 314	0.59	44.9±2.25
43	Isolate GASD 10	0.25	4.9±0.68
44	Isolate HKM 22	0.32	3.8±0.13
45	Isolate XLM 12	0.76	13.4±0.87
46	Isolate ORA 18	0.91	7.6±0.65
47	Isolate FPK 10	0.46	6.6±0.23
48	Isolate DPG 11	0.79	12.4±0.91
49	Isolate FDH 118	0.65	8.6±0.68
50	Isolate FDH 117	0.49	6.6±0.21
51	Isolate FDH 116	0.64	19.3±0.79
52	Isolate FDH 115	0.44	2.2±0.11

Data is expressed as mean±SD of triplicates

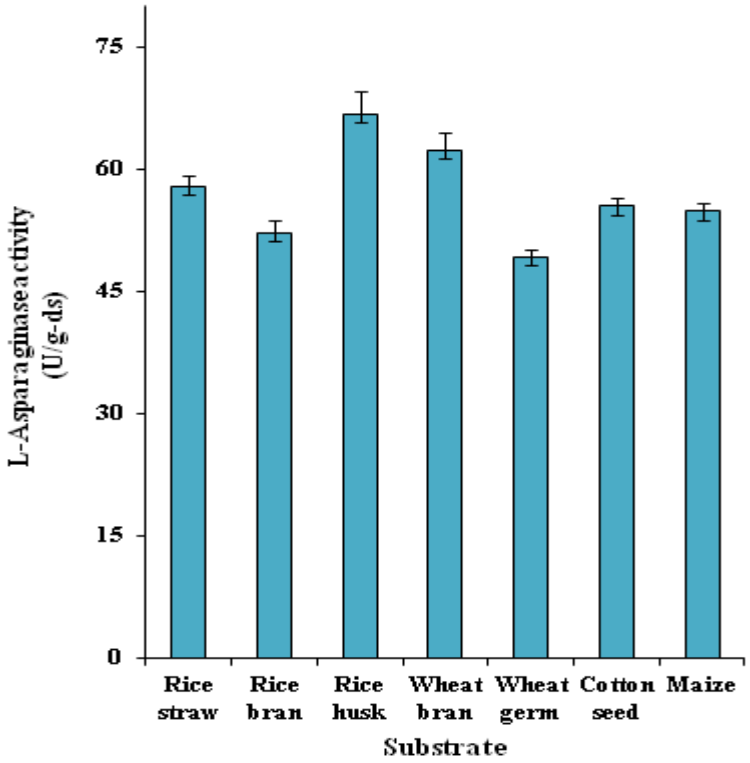

Fig. 2. Screening of different agro-industrial substrates for L-asparaginase production by *Trichoderma viride* F2 in SSF

3.5 Optimization of L-asparaginase Production under SSF

3.5.1 Effect of different physical factors on production of L-asparaginase

3.5.1.1 Incubation period

Effect of incubation period during the process of solid state fermentation was carried out for maximum L-asparaginase production. Analysis of culture supernatant showed enzyme activity rise from an initial of 16.26±0.76 U/g-ds after 24 h of incubation giving its peak activity of 74.67±3.27 U/g-ds on the fourth day of incubation (Fig. 3). By increasing the incubation period above this period a reduction in enzyme production was reported, which could be either due to the inactivation of the enzyme because of the presence of some kind of proteolytic activity or the growth of the organism might have reached a stage from which it could no longer balance its steady growth with the availability of nutrient resources. Moreover the heat that accumulates in the medium during mesophilic aerobic SSF due to poor heat dissipation could lead to a further drop in the oxygen level and thereby reducing the growth of the microrganism. Mishra [31] and Suresh and Raju [32] reported similar results for the production of L-asparaginase by *Aspergillus niger* and *A. terreus* MTCC 1782, respectively.

3.5.1.2 Incubation temperature

Temperature plays a very critical role in SSF as it ultimately affects the growth of the microorganism and has a profound effect on enzyme production. Maximum L-asparaginase production (73.58±2.19 U/g-ds) was reported at 28ºC (Table 2). The enzyme production reduced gradually with further increase in incubation temperature. The yield of L-asparaginase was drastically reduced at 40ºC (9.86±0.21), which may be due to the inactivation of microbial strain at higher temperatures due to the production of large amount of metabolic heat. Similar results were reported by Baskar and Renganathan [33] and Elshafei et al. [34] for L-asparaginase production by *Aspergillus terreus* and *Penicillium brevicompactum* NRC 829, respectively. The optimum temperatures for maximum enzyme

production was reported at 35ºC for *S. albidoflavous* [35], 40ºC for *A. niger* [31] and 30ºC for *Serratia marcescens* [36].

3.5.1.3 Moisture content

Optimum moisture content of substrate is necessary for proper growth of microbes as well as enzymes production. In the present research, maximal L-asparaginase production (84.68±3.69 U/g-ds) was recorded with 75% moisture content (Table 2). Furthermore, it was found that any deviation from the optimum humidity results in a decrease in enzyme activity, which can be attributed to the fact that higher moisture level decreases substrate porosity, promotes development of stickiness, reduction in gas volume, decreased gas exchange due to substrate particle agglomeration and reduced fungal growth and increases the chances of contamination [31,37-38]. Likewise, the low moisture level leads to sub-optimal growth, a lower degree of substrate swelling and higher water tension which decreases enzyme production [39].

3.5.1.4 Initial medium pH

Culture pH strongly influences many enzymatic processes and transport of various components across the cell -membrane, which in turn supports the cell growth and product formation. In the present study, maximum yield of L-asparaginase (90.23±3.87 U/g-ds) was reported at pH 5.0, while least enzyme production (53.24±1.71) was observed at pH 8.0 (Table 2). Similar pH was reported for the production of L-asparaginase by *Fusarium equiseti* as investigated by Hosamani and Kaliwal [40-41]. On the other hand, maximum enzyme production by *Bacillus subtilis* [38] and *Amycolatopsis* CMU-H002 [42] was observed at higher pH 7.0.

3.5.1.5 Inoculum level

Inoculum concentration was found to play an important role in L-asparaginase synthesis by *T. viride* F2. Under optimum conditions it was observed that there was a gradual increase in the synthesis of enzyme along with increase in the concentrations up to 1×10^8 spores/ml (113.43±5.11 U/g-ds). However, further increase above this level did not show any significant increase in the activity of L-asparaginase (Table 2). On the other hand, the lowest yield of enzyme (53.24±2.52 U/g-ds) was observed with an inoculum size of 1×10^4 spores/ml. The reduction of enzyme activity at inoculum sizes lower than optimum level can be attributed to few spores which lead to insufficient biomass. While, at higher inoculum sizes the decline in enzyme activity may be due to decrease in the concentration of the medium components [43].

Table 2. Effect of different physical parameters on extracellular L-asparaginase production by *Trichoderma viride* F2 in SSF

Incubation temp (ºC)	**L-asparaginase activity (U/g-ds)**
25	46.68±2.36
28	**73.58±2.19**
35	55.24±1.83
40	9.86±0.21
Moisture content (%)	**L-asparaginase activity (U/g-ds)**
50	62.91±2.78
66	75.21±3.24
75	**84.68±3.69**
80	67.74±2.92
83	48.66±1.87
86	46.93±1.34
pH value	**L-asparaginase activity (U/g-ds)**
3.0	57.18±2.96
4.0	69.73±3.21
5.0	**90.23±3.87**
6.0	82.14±3.35
7.0	61.52±1.87
8.0	53.24±1.71
Spores / ml	**L-asparaginase activity (U/g-ds)**
1×10^4	53.24±2.52
1×10^5	69.73±4.13
1×10^6	88.38±4.87
1×10^7	94.67±3.64
1×10^8	**113.43±5.11**
1×10^9	95.23±3.43
Surfactant (0.1 % w/v)	**L-asparaginase activity (U/g-ds)**
Control	111.15±3.23
Tween 20	**132.16±4.25**
Tween 60	106.26±3.24
Tween 80	114.15±4.21
Triton X-100	115.08±4.56
Tween 20 (%)	**L-asparaginase activity(U/g-ds)**
0.05	110.43±2.87
0.1	128.24±3.65
0.2	135.86±3.36
0.3	**141.15±3.76**
0.4	126.04±4.45
0.5	116.43±4.12
0.6	89.31±3.22

Data is expressed as mean±SD of triplicates

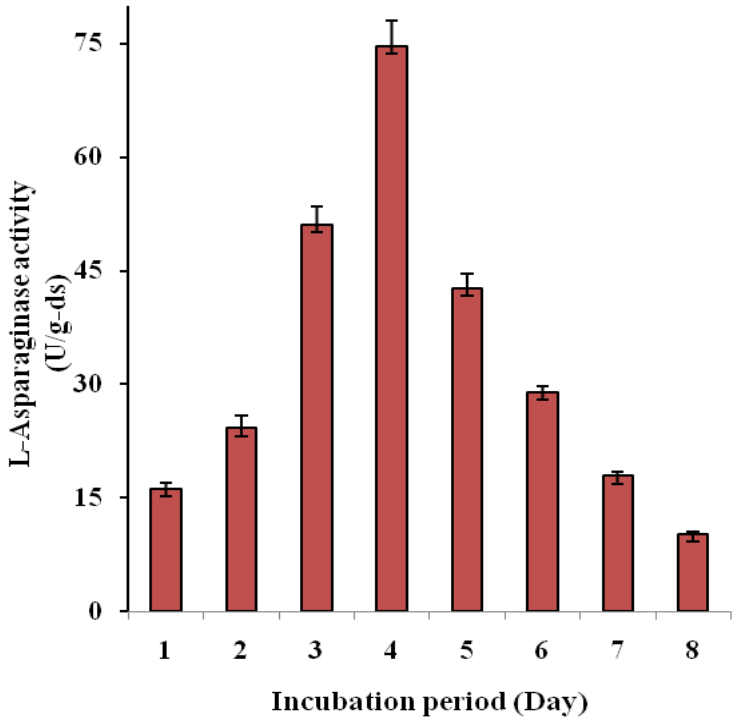

Fig. 3. Effect of different incubation periods on L-asparaginase production by *Trichoderma viride* F2 in SSF

3.5.1.6 Effect of different surfactants on L-asparaginase production

The addition of surfactants sometimes either increase or decrease enzyme production. Tween 80, Triton X-100 and related surfactants have been used for some time in bacterial cultures to assist in growth and also to promote the entrance of compounds into cells [44]. In the present study, each surfactant was added separately to study its effect on L-asparaginase production by *T. viride* F2. Presence of Tween 20 enhanced enzyme production by 1.19 folds (Table 2). While other surfactants did not impart much effect. In this concern, Sangeeth et al. [45] reported that, the addition of additives like sodium dodecyl sulphate (SDS), Triton X-100 and Tween 20 influenced protease and lipase production by *Bacillus licheniformis* VSG1. On the other hand, Triton X-100 and Tween 20 were found to decrease the secretion of amylase from *Bacillus* sp. as reported by Sudharshan et al. [46]. Different concentrations of Tween 20 were tried and maximum enzyme production (141.15±3.76 U/g-ds) was observed at 0.3% (w/v), while further increase in its concentration resulted in a reduction of L-asparaginase production (Table 2).

3.5.2 Effect of different nutritional factors on production of L-asparaginase

3.5.2.1 Influence of carbon source

The effect of various carbon sources on L-asparaginase production by *T. viride* F2 was studied and the result obtained was illustrated in Fig. 4. The medium without the tested nitrogen source served as control. Among all the carbon sources tested, glucose proved to be the best for L-asparaginase production yielding 156.38±4.57 U/g-ds followed by raffinose and soluble starch, while galactose and sucrose showed the lowest effect on L-asparaginase production. Glucose is commonly used as the primary carbon source for most of the microorganisms producing primary and secondary metabolites. Enhancement of L-asparaginase production by glucose was observed in *Aeromonas* sp. [47] and *Streptomyces ginsengisoli* [48]. Hymavathi et al. [49] reported that the most important carbon source for L-asparaginase production by *Bacillus circulans* was glucose, followed by mannose, while xylose and galactose were insignificant. Furthermore, glucose was found to be the best carbon source for L-asparaginase production by *Aspergilllus terreus* MTCC 1782 using modified Czapek`s Dox medium, followed by sucrose [50].

In mutants of *Serratia marcesens* namely mutant 933 and WF, enzyme production was improved by glucose and sucrose in mutant 933, while lactose inhibited enzyme production in mutant WF [51]. On the other hand, repression of L-asparaginase synthesis has been shown in bacteria such as *Serratia marcescens* [52] and *E. coli* [53]. Thus, the role of glucose in L-asparaginase synthesis remains controversial. Our results also showed that culture grown in 1.0% of glucose exhibited maximum enzyme production (Table 3), whereas at higher concentrations, glucose acts as a repressor for L-asparaginase production as reported earlier by Mukherjee et al. [54] for L-asparaginase production by *Enterobacter aerogenes* and similar trend was also observed in *Fusarium* sp. [55].

3.5.2.2 Effect of nitrogen source

A nitrogen source is a limiting nutrient and plays a key role in L-asparaginase production. Most of the microorganism utilize nitrogen source either inorganic or organic form or sometimes both. In the present work, the supplementation of additional nitrogen sources either organic or inorganic to the production medium had shown a profound impact on the production of L-asparaginase by *Trichoderma viride* F2 under SSF. Among the organic nitrogen sources tested, culture medium amended with casein favored maximum enzyme production (172.67±4.17 U/g-ds) followed by peptone (161.87±4.22 U/g-ds), while least enzyme production was detected in proline (107.32±1.87 U/g-ds) (Table 3). In this concern, Venil et al. [36] have reported peptone as the best organic nitrogen source for L-asparaginase production by *Serratia marcescens* SB08. On the contrary, Narayana et al. [35] reported yeast extract (2%) as the best nitrogen source for L-asparaginase production by *Streptomyces albidoflavus*. In the present work, the optimum level of casein for enzyme production was determined to be 1.5% w/v (186.56±4.56 U/g-ds), however further increase in casein concentration, resulted in a slight reduction in enzyme yield (Table 3). On the other hand, the data revealed that sodium nitrate as an inorganic nitrogen source was found to enhance L-asparaginase production from *T. viride* F2 with a yield of 193.32±4.01 U/g-ds, followed by ammonium nitrate (188.89±4.09 U/g-ds) (Table 3). These results are in congruence with that reported by Vuddaraju et al. [56], for L-asparaginase production by *Serratia marcescens* who found that $NaNO_3$ acts as a limiting nutrient and small variations in its concentration will alter either growth rate or product formation rate, or both, to a considerable extent. Different concentrations of $NaNO_3$ were tried and maximum enzyme production (215.13±4.16 U/g-ds) was observed at 0.15% (Table 3). Further increase in $NaNO_3$ concentration resulted in a reduction of enzyme production which may be due to the repressor effect of sodium nitrate at higher concentrations. Amena et al. [57] reported that ammonium sulphate at 0.25% was the best inorganic nitrogen source for L-asparaginase production by *S. gulbargensis.* While ammonium chloride was found to be the best nitrogen source for L-asparaginase production by *Aspergillus terreus* MTCC 1782 as reported by Baskar and Rangathan [33].

3.5.2.3 Influence of inducer on enzyme production

The enzyme yield was found to be maximized (223.72±4.39 U/g-ds) with L-asparagine at 0.1% w/v as shown in Table 3. While, L-glutamine had no effect on enzyme production. On the other hand, L-aspartic acid and L-glutamic acid had negative effect on enzyme yield. The reduction in enzyme yields (156.49±2.67 U/g-ds) by L-aspartic acid may be attributed to the feedback inhibition of enzyme production by L-aspartic acid.

3.5.2.4 Effect of various metal ion sources on l-asparaginase production

Metal ions are essential for cell mass formation and act as cofactor for several biosynthetic enzymes [58]. In the present work, different mineral ion sources were incorporated in optimized medium individually to determine their effect on asparaginase production. Results revealed that, the production of L-asparaginase was slightly enhanced by the presence of $MgCl_2$ (242.2±12.1 U/g-ds) followed by $MnCl_2$ (231.3±13.4 U/g-ds) and KCl (226.3±13.4 U/g-ds), while $AgNO_3$, $HgCl_2$, and $FeCl_3$ considerably inhibited the production (Table 4). Different concentrations of $MgCl_2$ were tried and maximum enzyme titer (276.5±13.4 U/g-ds) was observed at 0.05 % w/v, while a slight reduction in enzyme yield was reported with further increase in $MgCl_2$ concentration (Table 5).

Table 3. Effect of different nutritional factors on extracellular L-Asparaginase production by *Trichoderma viride* F2 in SSF

Glucose (%)	**L-asparaginase activity (U/g-ds)**
0.25	120.32±2.16
0.5	141.18±4.24
1.0	**152.21±4.52**
1.5	133.56±2.18
2.0	105.67±2.18
Organic Nitrogen (0.5 % w/v)	**L-asparaginase activity (U/g-ds)**
Control	148.48±3.28
Urea	143.73±3.12
Yeast extract	137.19±2.67
Casein	**172.67±4.17**
Malt extract	131.56±2.45
Proline	107.32±1.87
Peptone	161.87±4.22
Casein (%)	**L-asparaginase activity (U/g-ds)**
0.25	156.11±3.17
0.5	168.91±3.65
1.0	175.34±4.16
1.5	**186.56±4.56**
2.0	161.59±2.98
2.5	147.23±2.73
Inorganic Nitrogen (0.1 % w/v)	**L-asparaginase activity (U/g-ds)**
Control	181.46±3.35
Ammonium sulphate	176.54±3.67
Ammonium nitrate	188.89±4.09
Ammonium phosphate	171.23±4.34
Sodium nitrate	**193.32±4.01**
Ammonium chloride	170.12±4.18
$NaNO_3$ (%)	**L-asparaginase activity (U/g-ds)**
0.05	180.24±3.17
0.10	195.34±3.65
0.15	**215.13±4.16**
0.20	191.12±3.78
0.25	178.23±3.12
0.30	97.45±1.78
Inducer (0.1 %)	**L-asparaginase activity (U/g-ds)**
Control	211.21±3.54
L-Asparagine	**223.72±4.39**
L-Glutamine	203.56±4.12
L-Aspartic acid	156.49±2.67
L-Glutamic acid	178.27±2.45

Data is expressed as mean±SD of triplicates

Table 4. Effect of various metal ion sources on L-asparaginase production

Metal salt (0.01%)	**L-asparaginase activity (U/g-ds)**
Control	221.13±2.34
$AgNO_3$	57.44±0.74
$HgCl_2$	38.65±0.43
$FeCl_3$	76.41±1.53
$MgCl_2$	**242.21±12.10**
$ZnSO_4$	203.60±4.32
$CdCl_2$	191.93±2.78
KCl	**226.32±13.44**
$MnCl_2$	**231.34±13.42**
$CoCl_2$	214.56±11.21
KH_2PO_4	215.31±9.65
NaCl	201.28±7.39
$BaCl_2$	202.76±7.15

Data is expressed as mean±SD of triplicates

Table 5. Effect of different concentrations of $MgCl_2$ on L-asparaginase production

$MgCl_2$ (%)	**L-asparaginase activity (U/g-ds)**
0.005	227.27±9.18
0.01	238.34±11.64
0.03	254.53±10.27
0.05	**276.51±13.42**
0.06	269.26±14.38
0.07	260.78±12.76
0.08	248.15±9.53

Data is expressed as mean±SD of triplicates

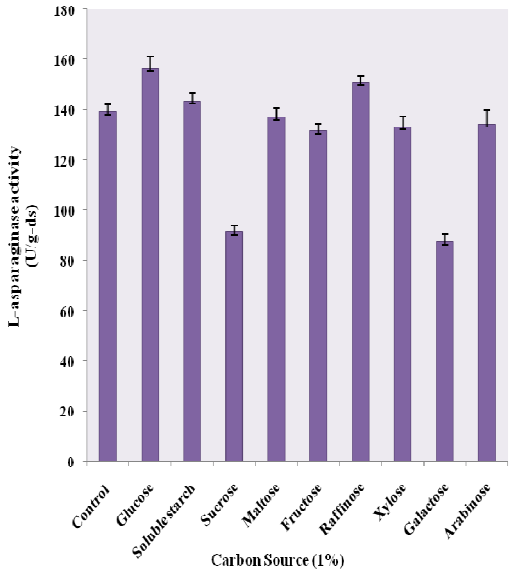

Fig. 4. Effect of different carbon sources on L-asparaginase production from *Trichoderma viride* F2 under SSF

4. CONCLUSION

Fifty-two fungal species and isolates were qualitatively and quantitatively screened for their abilities to produce extracellular L-asparaginase. From which, *Trichoderma viride* F2 exhibited the highest L-asparaginase production extracellularly using the low cost substrate, rice straw under SSF technique. Optimization of the cultural and environmental conditions required for maximum production of L-asparaginase was investigated. Rice husk (RH) followed by wheat bran (WB) exhibited the highest L-asparaginase production. The ratio of 3.0:2.0 of RH and WB gave the highest enzyme formation at initial moisture content of 75%, initial pH 5.0, and incubation period of 4 days at 28ºC. Presence of Tween 20 enhanced enzyme production by 1.19 folds. Glucose at 1.0% (w/v) was the best carbon source followed by raffinose and soluble starch. Casein at 1.5% (w/v) proved to be the suitable added organic nitrogen source for maximum enzyme production followed by peptone. While $NaNO_3$ at 0.15% was found to be the best inorganic nitrogen supplement for maximum L-asparaginase production. Furthermore, our results revealed that maximum enzyme yield was achieved with $MgCl_2$ at 0.05% (w/v) while, $AgNO_3$, $HgCl_2$, and $FeCl_3$ considerably inhibited the production. On the basis of the data obtained, *T. viride* F2 may be an unexploited source of potentially valuable products and may also be preferable to mass screening of common bacteria. This process can be amenable to large scale production and may be of interest to researchers and biopharmaceutical companies interested in developing and improving their therapeutic properties, which offer a great opportunity to scientific, biotechnological, economical, and industrial growth. Furthermore, developing an L-asparaginase production process based on rice husk and wheat bran as substrates in SSF is economically attractive due to the low cost and abundant availability of these raw materials in Egypt. Consequently, we suggest that enzymes which degrade amino acids should receive greater attention as potential therapeutic agents.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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