L-glutaminase production by *Trichoderma koningii* under solid state fermentation

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Abstract Solid state fermentation was conducted for the production of L-glutaminase by *Trichoderma koningii* Oud.aggr. using different agro-industrial byproducts including wheat bran, groundnut residues, rice hulls, soya bean meal, corn steep, sesamum oil cake, cotton seed residues and lentil industrial residues as solid substrates. Wheat bran was the best substrate for induction of L-glutaminase (12.1 U/mg protein) by *T. koningii*. The maximum productivity (23.2 U/mg protein) and yield (45.0 U/gds) of L-glutaminase by *T. koningii* occurred using wheat bran of 70% initial moisture content, initial pH 7.0, supplemented with D-glucose (1.0%) and L-glutamine (2.0% w/v), inoculated with 3 ml of 6 day old fungal culture and incubated at 30°C for 7 days. After optimization, the productivity of L-glutaminase by the solid cultures of *T. koningii* was increased by 2.2 fold regarding to the submerged culture.

Keywords L-glutaminase · Filamentous fungi · *Trichoderma koningii* · Solid state fermentation

Introduction

L-glutaminase (L-glutamine amidohydrolase E.C 3.5.1.2) is the enzyme that catalyze the deamidation of L-glutamine to L-glutamic acid and ammonia [1]. L-glutaminase has been received significant attention since it was reported extensively as antileukemic agent [2–4]. Unlike normal cells, leukemic cell do not demonstrate the L-glutamine synthetase, thus it dependent on the exogenous supply of L-glutamine for their growth and survival [5]. Therefore, blood L-glutamine serve as a metabolic precursors for nucleotide and protein synthesis of tumor cells [6]. Consequently, L-glutaminase causes selective death to L-glutamine dependent tumor cells by blocking the energy route for their proliferation [7]. Moreover, L-glutaminase could be used as efficient antiretroviral agent [8], biosensors for monitoring L-glutamine level [9] and in food industries imparting the flavor and aroma to the foods [10, 11].

L-glutaminase is ubiquitous in microorganisms including bacteria and fungi. L-glutaminase production by various bacterial genera under submerged culture conditions was extensively studied [12–14]. Furthermore, submerged cultures of *Verticillium malthousei* [13], *Aspergillus sojae* [15], *A. nidulans* [16], *Tilachlidium humicola* [17] and *Penicillium notatum* [18] had been used for L-glutaminases production. It appears from the documented literature a few reports being intensive, only, on the production of L-glutaminase by *Aspergillus oryzae* [19], *Beauveria* sp. [9] and *Zygosaccharomyces rouxii* [20] under solid state fermentation. It is worthy to note that, in spite of the broad advantages of the solid state fermentation bioprocesses that being less economically, needs small vessels, low moisture solid substrates were used, providing a selective environ-

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ment for growth and extracellular enzymes biosynthesis by filamentous fungi [21], it received less attention regarding to the submerged one. Thus, a searching for other fungal species for overproduction of L-glutaminase using agro-industrial solid substrates is pursued. Recently, solid state fermentation has been emerged as a promising bioprocess for production of enzymes and other economical metabolites on a large scale [20, 22].

In the present study, the productivity of L-glutaminase by *T. koningii* Oud. aggr. using solid agro-industrial residues as substrates was evaluated. In addition, the culture conditions as initial moisture content, initial pH, size of inoculum as well as the extra supplementation of carbon and nitrogen sources were optimized to maximize the enzyme yield. To the best of our knowledge, there is no reported publications deals with the production of L-glutaminase by *T. koningii* under solid state fermentation.

Materials and methods

Materials

L-glutamine, trichloroacetic acid, L-asparagine, L-methionine and Nessler reagent (HgCl_2 , KI and NaOH) were purchased from Sigma Chemical (St. Louis, Mo. USA). Glycine was obtained from PROLABO (Paris, France). Folin reagent was purchased from LOBA (Mumbai, India). All other chemicals used were of analytical grade.

Microorganism

Twenty fungal isolates were recovered from the soil of Sharkia province, Egypt, on L-glutamine containing medium (unpublished data). L-glutamine-Dox's medium [16] that employed for isolation of glutaminolytic fungi contains (g/L); L-glutamine 10.0, D-glucose 2.0, KH_2PO_4 1.50, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, KCl 0.5, $\text{CuNO}_3 \cdot 3\text{H}_2\text{O}$ 0.02, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02 and agar-agar 20.0, dissolved in 1 litre distilled water (pH 6.5). *Trichoderma koningii* Oud. aggr. had the most glutaminolytic activity among the twenty isolated fungal species. Consequently, *T. koningii* was selected in the subsequent experimentation for L-glutaminase production using solid substrates. The fungal cultures were maintained on potato-dextrose agar (Oxoid LTD., Hants, England), incubated at $28 \pm 1^\circ\text{C}$ for 7 days then stored at 4°C until use.

Conidial suspension were prepared from a freshly raised 7 day old culture of *T. koningii* on potato-dextrose agar slants by suspending in 10 ml of 0.85% sterile saline solution.

Fermentation medium and culture conditions

Several agro-industrial byproducts namely; wheat bran, groundnut residues, rice hulls, soya bean meal, corn steep liquor, Sesamum oil cake, cotton seed residues, lentil industrial residues were utilized as substrates for L-glutaminase production by *T. koningii*. Five grams of each substrate, after drying in mechanical dryer at 50°C till constant moisture content, were dispensed into 250 ml Erlenmeyer conical flasks and moistened with 10 ml of salt solution containing glucose 0.6%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05% and KCl 0.05%. The flasks were autoclaved at 121°C for 25 min, cooled to room temperature and inoculated with 2 ml of the fungal conidial suspension which prepared previously. The inoculated flasks were mixed thoroughly and incubated at 30°C for 8 day in static incubator (New Brunswick Scientific Edison NJ, USA).

Optimization of the culture condition for L-glutaminase production

The different physicochemical parameters to maximize the yield of L-glutaminase by *T. koningii* under solid state fermentation were investigated. The optimized parameter was incorporated at its optimized level in the subsequent optimization experiments. The impact of initial moisture content (20–90%), initial pH (3–11, adjusted with 1N HCl or 1N NaOH), incubation temperature (15 – 50°C) and size of inoculum on L-glutaminase production using solid-cultures of *T. koningii* was evaluated. Moreover, the effect of incorporation of additional carbon sources (glucose, arabinose, mannitol, sorbitol, fructose, cellulose and starch at 1% w/v), additional nitrogenous compounds (NaNO_3 , NH_4Cl , yeast extract, casein, beef extract, L-asparagine, L-methionine, $(\text{NH}_4)_2\text{SO}_4$, malt extract and L-glutamine at 1% w/v), in addition to the effect of different doses of L-glutamine (0.2–5% w/v) to the production medium were studied. All the experiments were conducted in triplicate and the mean values are considered. After incubation of each fermentation sample, the crude enzyme was prepared.

Crude enzyme extraction

The crude L-glutaminase was extracted from the fermented solid substrate by applying a simple contact method using citrate-phosphate buffer (pH 7.0) according to the method of Kashyap et al. [20]. The fermented solid substrate was mixed thoroughly with 40 ml of the buffer (pH 7.0) using a rotary shaker 200 rpm for 30 min. The entire contents of the flask were squeezed through a cheese cloth, the pooled extract were centrifuged at 4°C for 20 min at 10000 rpm.

The clear supernatant was collected and used as the crude enzyme for subsequent assays.

L-glutaminase assay

L-glutaminase was assayed by direct Nesslerization according to Imada et al. [13]. The enzymatic reaction mixture contains 1 ml of 1% L-glutamine in citrate-phosphate buffer (pH 7.0) and 1 ml of the crude enzyme incubated at 30°C for 1 h. The enzymatic activity was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid. The reaction mixture was centrifuged at 5000 rpm for 5 min to remove the precipitated protein. The released ammonium was determined using 0.5 ml Nessler reagent, after 15 min the developed color was measured at 480 nm using a spectrophotometer (Spekol-Spectrocolorimeter). Enzyme and substrate blanks were used as controls. The ammonium concentration of the reaction was determined by inference from the standard curve of ammonium sulphate. One unit (U) of L-glutaminase was defined as the amount of enzyme that liberates 1 μ mol of ammonia under optimal assay conditions. Specific activity of L-glutaminase was expressed as the activity of the enzyme (U) per mg protein released. Enzyme yield was expressed as the activity of L-glutaminase per grams dry substrate (U/gds).

Determination of protein concentration

The concentration of protein of the crude enzyme was estimated by Folin reagent according to the protocol of Lowry et al. [23], using bovine serum albumin as standard. The protein concentration was expressed by mg/ml of crude enzyme.

Results and discussion

Twenty fungal isolates have the potentiality to grow on medium containing L-glutamine as the sole nitrogen source as it appeared from the preliminary screening experiment (data not shown). The maximum productivity of L-glutaminase was quantified in the submerged culture filtrate of *T. koningii* (10.3 U/mg protein) that justified it for selection during the subsequent experimentation under solid state fermentation.

The potential economical advantages of solid state cultures may include a reduced thermal processing requirements, reduced energy requirement for agitation with high extracellular product concentration [24]. Solid substrates utilized are insoluble in water and employed as carbon, nitrogen, minerals as well as growth stimulators. Unlike bacteria and yeasts that grow by surface adhering to solid

substrates, filamentous fungi have the potentiality to deeply penetrate the solid particles for nutrient up taking.

The screening for L-glutaminase production by *T. koningii* using different solid substrates (Fig. 1) show that, a detectable variation of L-glutaminase productivity regarding to the type of employed substrate. The maximum L-glutaminase productivity (12.1 U/mg protein) and yield (23.4 U/gds) by *T. koningii* were observed using wheat bran, followed by cotton seed residues, sesamum oil cake and corn steep as cultural substrates. Otherwise, the lowest enzyme productivity by *T. koningii* was measured in the presence of rice hulls followed by soya bean meal, groundnut residues and lentil industrial residues as solid substrates. The solid cultures of *T. koningii* using wheat bran may display the highest yield of enzyme, this may be due to the excellent mechanical properties of wheat bran (structure retention and lack of particle agglomeration) in addition to their good nutritional value that stimulates the fungal sporulation. These observations are in agreement with those observed for *Aspergillus oryzae* [17, 25] and *Zygosaccharomyces rouxii* [20].

The obtained data in Fig. 2 reveals the significant effect of initial moisture content of wheat bran as solid substrate on the enzyme productivity by *T. koningii*. It could be seen that, the maximum L-glutaminase productivity (12.5 U/mg protein) and enzyme yield (24.13 U/gds) were observed at 70% initial moisture content. A further increase in the initial moisture content resulted in existence of free water and consequently a significant reduction on both enzyme productivity and yield by *T. koningii* was detected. The inhibitory effect on fungal productivity by higher moisture content may be due to the substrate particle agglomeration, lower O₂ transfer, decreasing of porosity and enhancement of bacterial growth. These results are coincide with those previously reported for L-glutaminase production by *Vibrio costicola* [26] and *Z. rouxii* [20].

The maximum L-glutaminase productivity (12.5 U/mg protein) and enzyme yield (24.13 U/gds) by solid cultures of *T. koningii* using wheat bran were recorded at pH 7.0 (Fig. 3), also it could be concluded that *T. koningii* able to survive over a much wider pH range (pH 6–8). L-glutaminase activity was drastically decreased at pH below 4.0 and above 9.0. The optimal L-glutaminase productivity by *T. koningii* was determined at the neutral pH, this may attributed to the balance of ionic strength of plasma membrane [27]. These results are in coincidence with that reported for L-glutaminase production under solid state fermentation using *Vibrio costicola* [26]. Sabu et al. [9] reported that, the maximum L-glutaminase productivity by *Beauveria* sp. under SSF had two pH optima, one at pH 6.0 and another at pH 9.0.

Fig. 1 Production of L-glutaminase by *Trichoderma koningii* using different solid substrates

Fig. 2 Effect of initial moisture content of wheat bran on L-glutaminase production by *Trichoderma koningii*

The data in Fig. 4 clearly reveals the significant effect of inoculum concentration on L-glutaminase production by *T. koningii* under solid state fermentation. A gradual increasing on both productivity and yield of L-glutaminase of *T. koningii* with inoculum size till reaching to their optimal values (18.8 U/mg protein and 35.0 U/gds) at 3 ml spore of 6 day old culture. With further increasing in the concentration of

inoculum (5 ml) a detectable reduction on the enzyme productivity could be seen, that might be attributed to nutrient limitation [20] or accumulation of some non-volatile self inhibiting substance [28]. Otherwise, with lower inoculum size (0.2 ml) a measurable decreasing of both enzyme productivity (6.10 U/mg protein) and yield (13.01 U/gds) were registered, that could be due to the lesser number of cells

Fig. 3 Effect of initial medium pH on L-glutaminase production by *T. koningii* under SSF

Fig. 4 Effect of inoculum concentration on L-glutaminase production by *T. koningii*

that require a long time to grow for utilizing the substrate and forming the desired products [29].

Incubation temperature has a profound effect on L-glutaminase production by *T. koningii* under solid cultural conditions (Fig. 5). The maximum enzyme productivity (18.8 U/mg protein) and yield (35 U/gds) by the experimental fungal isolate were obtained at 30°C. However, the enzyme productivity by *T. koningii* was reduced gradually with further incubation temperature, above optimal. At 50°C

the enzyme yield was declined by about 77.15% regarding to that at optimum temperature. In agreement with these results, those obtained for L-glutaminase production by *A. oryzae* [25] and *Zygosaccharomyces rouxii* [20]. Likewise, the optimal temperature for L-glutaminase production by *T. koningii* being lower than that reported for *Vibrio costicola* [26] and *Lactobacillus rhamnosus* [30].

The incorporation of additional carbon sources (Fig. 6) to the basal solid-state fermentative medium of *T. koningii*

Fig. 5 Effect of incubation temperature on L-glutaminase production by *T. koningi*

Fig. 6 Effect of different carbon sources on L-glutaminase production by *T. koningi*

exerted a considerable effect on the biosynthesis of L-glutaminase. A slightly stimulatory effect was observed on L-glutaminase production by the fungus using glucose (35 U/gds) followed by arabinose (30 U/gds) and fructose (27 U/gds) comparing to the carbon free medium. While starch, mannitol and cellulose may retard the enzyme bio-

synthesis by the solid culture of *T. koningi*. The slightly stimulatory influence of glucose that approximated by 31.5% regarding to glucose-free wheat bran basal medium may be attributed to the positive effect of glucose as a cometabolic agent or due to the efficiency of the fungus to utilize the wheat bran as carbon, nitrogen and miner-

Fig. 7 Effect of supplementation of nitrogen sources on L-glutaminase production by *T. koningii*

als source [31]. Similar results were reported, proved the stimulatory effect of glucose on L-glutaminase biosynthesis under solid state fermentation by *Vibrio costicola* ACMR267, *Beauveria* sp. BTMFS 10 and *Z. rouxii* [26, 9, 20].

The supplemental nitrogenous compounds (Fig. 7) viewed the significant conduction of L-glutaminase by *T. koningii* under solid state fermentation. It could be concluded that all the additional nitrogen sources had a negative effect on the biosynthesis of L-glutaminase using the solid-fungal cultures except L-glutamine that enhance the enzyme yield by about 12.0% comparing to the control (nitrogen-free medium). The maximum activity of L-glutaminase (23.2 U/mg protein) and yield (45.0 U/gds) by *T. koningii* were obtained by incorporation of 2% L-glutamine to the wheat bran basal productive media. The repressor effect of all nitrogenous sources on enzyme production by the experimental fungal isolate could be ascribed to the preferential utilization of these nitrogen nutrients comparing to the wheat bran. Thus, the enzyme production being not justified by the solid fungal culture in the presence of these organic and inorganic nitrogenous compounds other than L-glutamine. It noteworthy that L-glutaminase production by *T. koningii* may be inductive, these observations seem to be confirmed by those reported

for other microbial glutaminase under solid state fermentation bioprocessing [9, 26].

Conclusion

The current study was focused on the evaluation of the potentiality of *Trichoderma koningii* Oud. aggr. for utilization of different agro-industrial byproducts namely; wheat bran, groundnut residues, rice hulls, soya bean meal, corn steep, sesamum oil cake, cotton seed residues and lentil industrial residues as substrates for production of L-glutaminase under solid state fermentation. L-glutaminase an enzyme of super therapeutic and technological values that could be sustained by conventional lower economical/higher efficiency productive bioprocesses. Therefore, solid state fermentation seems to be the prospective technique for large-scale production of microbial metabolites of biotechnological importance.

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