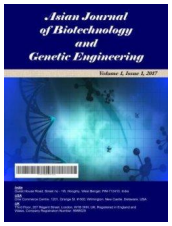

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New Documentary of Acid-Stable Glutaminase Production by an Efficient Acidophilic *Aspergillus niger* CPGM 1439

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Authors' contributions

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

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ABSTRACT

Samples for isolation were taken from natural spoiled salted foods. All acidophilic isolates were subjected under comparison for finding the most potent glutaminase producing one. Optimization of the produced enzyme was done in the light of seven environmental and five nutritional factors including incubation time, temperature, pH, buffer system, static and different shaking speeds conditions, dark and light conditions, different inoculum size, additional carbon sources, additional nitrogen sources, heavy metals, vitamins, amino acids. Five fungal isolates have been selected and were tested for their ability to grow on L- glutamine containing medium at pH 3 and 2. Only 5 fungal isolates (F1.a, F2.a, F3.a, F4.a and F5.a) were able to grow efficiently on pH3, two of them were able to grow also on pH2. The activity of enzyme was measured extracellularly and intracellularly for each isolate and were 20.34, (2.68), 17.8, (0.423), 5.77, (5.77), 10.27, (1.39), 2.28 and (0.071) U/mL respectively. The highest enzyme activity producing isolate (F1.a) was selected and identified

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genetically. It was found to be closely related to *Aspergillus niger* strain CPGM 1439 with 93% homology and accession number DQ196192.1. The yield was quiet high after optimization reached 49.59 U/mL extracellularly which is essential advantage for such production method. The maximum yield was obtained at 35°C, pH1.8, 100 ppm Mn^{+2}, addition of iso- leucine 3.59% for 7 days incubation in dark condition and shaking speed 100 rpm. Glutaminase enzyme was produced efficiently by the finally selected acidophilic *Aspergillus niger* isolate and optimization lead to higher enzyme activity which was measured by U/mL in each step.

Keywords: Glutaminase; acidstable; enzyme activity; optimization; extracellular and intracellular.

1. INTRODUCTION

L- glutaminase (L-glutamine amidohydrolases E.C. 3.5.1.2) catalyses the hydrolysis of L-glutamine to glutamic acid and ammonia [1]. This enzyme has wide range of economic importance as it can be used in food industry as tool for hydrolysis of protein because of its action in conversion of L- glutamine to ammonia and glutamic acid which gave the special flavor to food products [2] also, it is nowadays the core of many researches to be used as therapeutic agent for cancer by searching for potential and selective inhibitors for this enzyme as it regard central enzyme for many conversions occur in the cell which lead to increase proliferation of cancer cells [3]. Moreover, L-glutaminase is applied in the therapy of human immunodeficiency virus (HIV) [4] and could be used as efficient antiretroviral agent [5]; L-glutaminase biosensors for monitoring L-glutamine level [6] and in food industries imparting the flavor and aroma to the foods [7,8].

There is great trend to produce L-glutaminase enzyme with unique stability properties to withstanding harsh industrial conditions and retain its activity under such harsh industrial processes. L- glutaminase can be found in different types of organisms [9] including bacteria [10], fungi [11], yeast [12], Actinomycetes [13] and can also extracted from plants [14].

It was reported that, there are many factors that can affect the production of glutaminase enzyme from any microorganism which differ according to the nature of the producer microorganism as pH, temperature, incubation time, additional substances or vitamins of metals of natural supplements can greatly cause great influence on the enzyme yield, optimization of production is very important shotgun point [15].

The main aim of this study is synthesizing glutaminase enzyme biologically using microorganisms which produce the enzyme efficiently in their medium or intracellularly without any possible problems during their growing time and with high productivity. Also, main point in the produced enzyme is that to have tolerance to high acidic medium which make it superior enzyme which gives a chance to use it in the proposed applications with high quality.

2. MATERIALS AND METHODS

2.1 Isolation

Isolation was carried out on this medium: The medium was composed of (g/l) NaCl 10, $MgSO_4.7H_2O$ 10, K_2HPO_4 10, Glutamine 10 and Agar 20. The pH of the medium was adjusted at 2 and 3. Incubation was for five days at 30°C, the isolates which were able to be sub-cultured with high growth were selected and purified.

2.2 Separation of the Extracellular and Intracellular Crude Enzyme

The production was done primarily in 250 ml conical flask containing 50 ml broth medium having the same constituent as that for isolation except agar. Sterilization was carried out and inoculation with 1ml of spore suspension of each isolate. The extracellular crude enzyme was separated by filtration and the mycelium was washed with distilled H_2O. The intracellular contents of the cells were obtained by ultrasonic disruption of cells by ultrasonic processor (Cole Parmer Ultrasonic Homogenizer CPX 400) over three 15 s periods, and with an interval of 45 s between periods. The sonicated samples were centrifuged at 15,000 rpm for 30 min at 4°C to remove cell-debris using Sorvall cooling centrifuge. The supernatants were then used for assay of enzyme [16].

2.3 Quantitative Assay of Glutaminase Enzyme

The assay was done according to Imada et al. [17]. First the reaction mixture was prepared by

adding 0.5 m1 of the sample, 0.5 mL of 0.04M L-glutamine solution, 0.5 m1 of distilled water and 0.5 m1 of phosphate buffer 0.1M, pH 8.0. The mixture was incubated at 37°C for 15 min then, 0.5 mL of 1.5 M Trichloroacetic acid was added for stopping the reaction. After that, 0.1 m1 of the mixture was added to 3.7 mL of distilled water and finally 0.2 m1 of Nessler's reagent were added to complete the volume to 4 mL. the same steps were done for controls. The absorbance was measured at 450 nm using a UV -Visible spectrophotometer. The absorbance reading was indication for ammonia liberated from the action of the enzyme. Standard curve using ammonium chloride was made using serial dilutions of ammonium chloride covering nearly all the range which had been obtained. The enzyme yield was expressed as Unit/mL (U/mL).

It's known clearly that, one international unit of L-glutaminase was defined as the amount of enzyme that liberates one micromole of ammonia under optimum conditions.

3. RESULTS AND DISCUSSION

3.1 Isolation of Acidophilic Glutaminase Producing Microorganisms

In the present investigation the starting basic point is searching for new sources for isolation of L- glutaminase producers from acidic environment to obtain novel isolates with special characters. In this respect 5 fungi were isolated at pH 3 (F1.a, F2.a, F3.a, F4.a and F5.a) from pickled lemon, pickels, lemon and salted cheese and only two isolates were able to grow at pH 2 (F1.a and F2.a). The five fungal isolates were assayed extracellularly and intracellularly for glutaminase production as recorded in table (1), Isolates F1.a followed by F2.a were the most producers extracellularly and recorded 20.34 and 17.80 U/mL, respectively. In addition, the intracellular enzymes produced by the five fungal isolates have moderate productivity compared with the extracellular glutaminase. Extracellular glutaminases are more advantageous over intracellular one as they could be produced economically industrially in the nutrient medium and purified easily; this was in full agreement with the results of [18].

A variety of microorganisms, including bacteria, yeast, and filamentous fungi, from both soil and marine habitat have been reported to produce L-glutaminase. In this aspect, many researches have been reported isolation of glutaminase producers from soils as [19] who isolated 20 fungi and tried to produce glutaminase. Also, [20] isolated a soil bacterium on Zobell's agar medium which was used for screening and maintenance of the bacterial culture. In addition, several studies have been started using salt lakes or water in general as the source of the isolation as reported by [15] who obtained his isolate from marine sediment of coastal environment growing on mineral salt glutamine medium as the medium for production [21] while culture maintenance was on Bennet' agar medium [22].

3.2 Optimization of Glutaminase Productivity for Selected Acidophilic Isolate (F1.a)

seven environmental and five nutritional factors were carried out including incubation time, temperature, pH, buffer system, static and different shaking speeds conditions, dark and light conditions, different inoculum size, additional carbon sources, additional nitrogen sources, heavy metals, vitamins, amino acids.

Concerning the incubation time for the glutaminase production by isolate F1.a, the best production time was from 6 - 8 days with the best at 7^{th} day, the reason for decreasing in the enzyme yield after 8^{th} day might be attributed to the inactivation of glutaminase by the protease secreted by the fungus as reported by Zhu et al. [23]. While the optimum incubation temperature was 35°C and the best inoculum size used was $2.48x10^6$ spore/mL and any further increase above this level resulted in a marginal decline in the enzyme production. These results were in full agreement with that of Hamed and Alwasify [24] who reported that the maximum yield of glutaminase by *Fusarium oxysporum* was achieved at 35°C, and after 7 days of incubation using inoculum of 100 µmL / 30 mL medium. Also, El-Sayed [19] reported the best production was at 30°C and 7days of incubation and inoculum size was 3mL (mL/wt) for L-glutaminase produced by *Trichoderma koningii.*

In the present investigation, the effect of light and dark as well as speed of agitation on enzyme production was also studied. Dark was preferred by the selected fungal isolate and 100 rpm was also result in activation of the productivity of L-glutaminase (No literatures were available concerned with this this point of research with glutaminase production).

This is the first documentary to produce L-glutaminase by acidophilic microorganisms. Isolate F1a. can live and produce L-glutaminase at different acidic pH's ranged from 1.5 up to 5 (Table 2) by adjusting the pH using 1N HCl but it scored maximum productivity at 1.5 followed by 2.0 with 28.55 and 26.20 U/mL of enzyme productivity, respectively. Probably the isolate could be assigned as acidophilic with reference to enzyme production. By using different acidic buffer systems, Hydrochloric Acid-Potassium Chloride buffer was best buffer system used (25.93 U/mL) but it still less than that adjusted by the 1NHCl. In view of the findings of other investigators, few reviews were found concerned with the production of L-glutaminase with microorganisms at lower pH, only pH range from 4-6 was used in this respect as showed by Krishnan et al. [25], pH 4.1 [26], pH 5.0 by *Aspergillus oryzae* S 2; [27], pH 4 by *Aspergillus* sp. ALAA-2000. The present isolate has the ability to produce L-glutaminase at pH 1.5, so the produced L-glutaminase must be acido-stable and can stand the acidity of the HCl in the stomach if used in food industry.

Table 1. Quantitative assay of glutaminase for selected acidophilic isolates

Isolate code	Production type	Released ammonia (M)	Enzyme productivity (U/mL)
F1.a	Extracellular	0.02034	20.34
	Intracellular	0.00268	2.68
F2.a	Extracellular	0.0178	17.80
	Intracellular	0.000423	0.423
F3.a	Extracellular	0.00577	5.77
	Intracellular	0.00577	5.77
F4.a	Extracellular	0.01027	10.27
	Intracellular	0.00139	1.39
F5.a	Extracellular	0.00228	2.28
	Intracellular	0.0000706	0.071

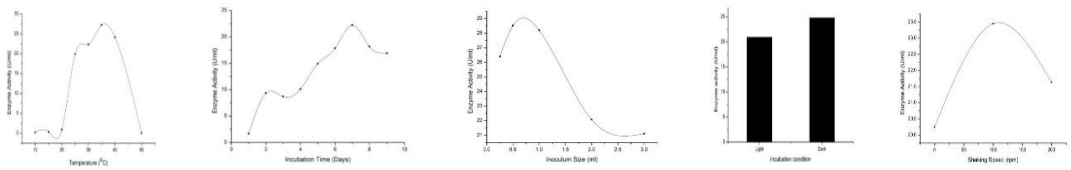

Fig. 1. Effect of different incubation time, temperature, inoculum size, dark and light and agitation speed on glutaminase productivity by isolate (F1.a)

Table 2. Effect of different pH values and different buffer systems on glutaminase productivity for isolate F1a

(a) Different pH vales

pH	Final pH	Enzyme productivity (U/mL)
1	1.7	0.749
1.5	5.3	28.55
2	6.2	26.2
2.5	6.3	19.37
3	6.2	17.45
3.5	6.5	15.97
4	6.4	15.5
4.5	6.4	14.61
5	6.9	15.07

(b) Different buffer systems

Buffer system	pH	Enzyme productivity (U/mL)
HCl-KCl	1	1.61
	1.2	1.85
	1.4	7.86
	1.6	19.37
	1.8	25.93
	2	13.04
	2.2	8.59
Phthalate-NaOH	2.2	13.49
	2.4	10.35
Glycine- HCl	2.2	7.69
	2.4	9.24

Table 3. Effect of different concentrations of K_2HPO_4, NaCl and $MgSO_4.7H_2$ on L-glutaminase productivity by the most potent fungal isolate

K_2HPO_4 (%)	Enzyme productivity (U/mL)	NaCl (%)	Enzyme productivity (U/mL)	$MgSO_4.7H_2O$ (%)	Enzyme productivity (U/mL)
0.5	13.59	0.5	1.25	0.5	19.31
1	28.56	1	28.56	1	28.56
2	22.72	2	20.86	2	23.69
3	23.19	3	22.77	3	0.52

In case of studying the effect of different glutamine concentration as a substrate for the production of L-glutaminase by the most potent acidophilic fungal isolate F1a., a concentration of 2% was exhibited the highest productivity, this result was in complete accordance with the results of several authors [28,19,29] who proved that a 2% of glutamine was enough to produce the highest L-glutaminase by *Pseudomonas* sp. BTMS-51; *Trichoderma koningii* and *Vibrio azureus* JK-79, respectively. On the other hand, [6,30] reported that, only 0.25 and 0.5% glutamine was enough in the production of L-glutaminase, respectively. While, [31] found that, 1% glutamine exhibited the highest L-glutaminase activity by *Stenotrophomonas maltophilia* NYW-81. Moreover, a concentration of 3% glutamine proved to produce the highest L-glutaminase activity as reported by Revanth and Raju [32] and maltose as reported by Kiruthika and Saraswathy [29].

Table 4. Effect of different glutamine concentrations on enzyme productivity for acidophilic isolate (F1.a)

Glutamine (%)	Enzyme activity (U/mL)
0.25	7.44
0.5	15.12
1	25.25
2	39.25
3	28.95

In the present investigation, incorporation of additional carbon sources doesn't enhance any increase in the L-glutaminase productivity compared with control group, but on the other hand inhibit the productivity. In view of the findings of the other workers, [33] stated that, many sources utilized found to be glutaminase inhibitors like fructose, sucrose furthermore, lactose and starch were also found to have inhibitory effect on glutaminase reported by [34] while enhancing has been approved on addition of glucose [35,6,28,19,36,37], sucrose [30,32], maltose [38], sorbitol [31], galactose [39].

In the present study, all the nitrogen sources organic and inorganic tested along with L-glutamine resulted in a decrease in L-glutaminase yield compared to control. The results indicated very clearly that addition of additional nitrogen sources in did not enhance enzyme production, and contradictory these additional nitrogen sources inhibited (Table 4) L-glutaminase synthesis by the acidophilic fungus which was in compatibility with [33] urea and peptone were recorded as inhibitors for glutaminase. Testing adding different nitrogen sources were concerning other researchers as [40] who found that, addition of NH_4NO_3 or NH_4Cl enhanced the activity of glutaminase while KNO_3 decreased or has no effect under certain conditions typically with [41] NH_4Cl also was found as glutaminase enhancer [42]. Some researchers reported that, on supplementation with additional nitrogen compound the activity increased as yeast extract [39], malt extract [38] peptone and sodium nitrate [34].

This variability refereed to the behavior of the producing microorganism for the present additional carbon or nitrogen source basically the presence of specific enzymes. Also, it was proved that, carbon may result in increasing N compound uptake and transport changing the behavior of microorganism for the production of glutaminase the core of the research [43].

Some enzymes may require the presence of specific metal for their activation or for increasing their activity as the microorganism can use this metal as electron donor or acceptor deriving the energy required for the microorganism thus, six different metal containing compounds were used at three different concentrations 50, 100 and 200 ppm of the metal testing their effect on glutaminase productivity. the acidophilic isolate f1.a showed maximum activity 39.93 U/mL at 100 ppm Mn^{+2} as manganous sulphate comparing to control 39.25 the other tested six metal caused inhibition of the enzyme mainly $HgCl_2$ and $CdSO_4$ inversely affected the glutaminase activity with

decreasing badly. This may be due to the hazardous effect of Hg and Cd on the isolate growth in the first place and so its ability to survive and producing the enzyme owing to the fact that, not all microorganisms able to synthesizing the enzymes that can help them in detoxifying the hazardous toxic metals [44]. Kumar et al. [39] paid out in research that, glutaminase reached maximum activity in the in the presence of $MnSO_4$ fitted with the results of this research. While in another research the isolate showed maximum glutaminase activity 32.5 U/mL in the presence of $ZnCl_2$ in concentration 1 w/v [45]. Also, magnesium was reported as metal activator for the glutaminase by *Aspergillus wentii* MTCC 1901 as magnesium sulphate [46] or as magnesium chloride [27]. Many other metals were incorporated and were found as glutaminase inhibitors as Li, Ca, Ba [27].

Concerning the effect of vitamins on glutaminase, seven vitamins were used with different concentrations (50, 100 and 200 ppm). None of the used vitamins has enhancing impact on glutaminase productivity otherwise all tested vitamins caused inhibition of the enzyme. Vitamins role in microorganisms are cofactor may aiding in metabolism of amino acids or proteins and can increase the activity of the produced enzyme depending on the producing microorganism which interfering in its metabolic cycles [47].

In case of supplementation with additional different amino acids together with the glutamine presence, Isoleucine played an important role as inducer for L-glutaminase synthesis for this acidophilic fungus under study followed by Alanine and Aspartic acid with glutaminase activity reached 49.59, 44.01 and 38.15, respectively while, the rest of amino acids were found inhibitory for L-glutaminase productivity (table 5) by the acidophilic fungus. However, [27] reported the production increased in the presence of cysteine. In addition, methionine caused increasing in glutaminase activity [22]. L-glutamic acid, l- aspartic and l-asparagine were recorded as glutaminase activity improvers [48] in contrast to Chitanand and Shete [49] asparagine and other tested amino acids (l-tyrosine and l-lysine) were found to be glutaminase inhibitors.

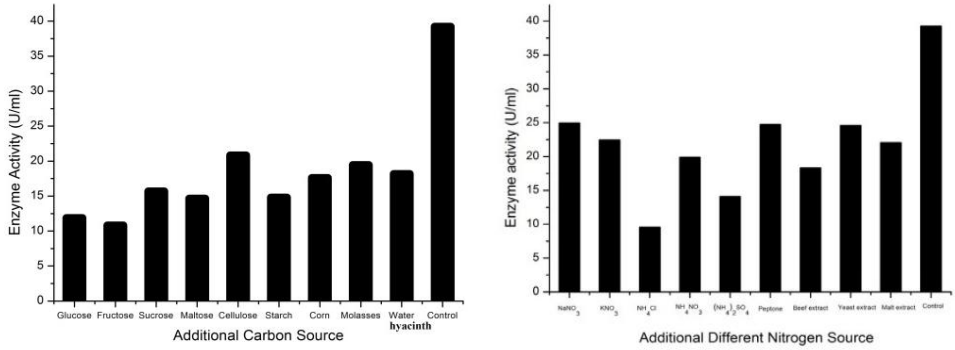

Fig. 2. Effect of additional different carbon and nitrogen sources on enzyme productivity for acidophilic isolate (F1.a)

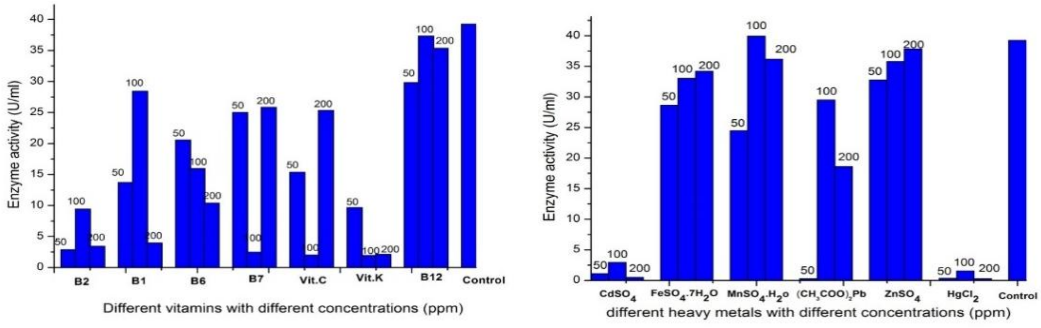

Fig. 3. Effect of different (a) Vitamins and (b) heavy metals with different concentrations on glutaminase productivity by acidophilic

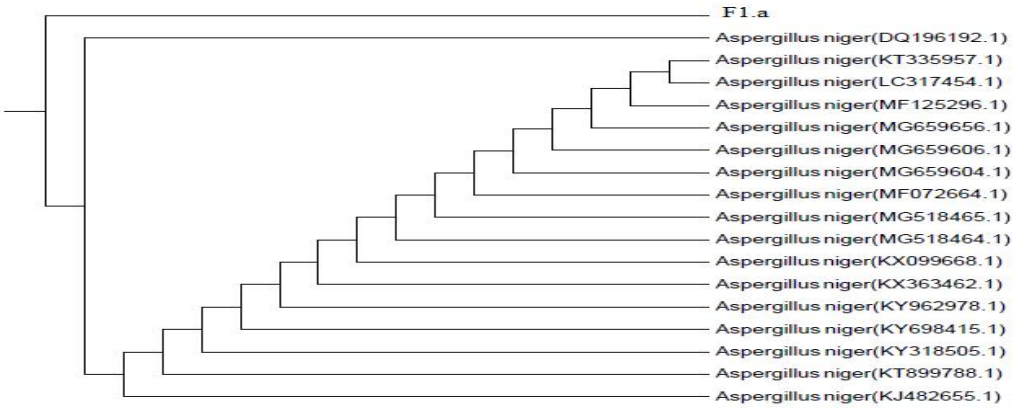

Fig. 4. A dendrogram showing the sequence relationships between *Aspergillus niger* CPGM 1439 and several other strains based on 18S ribosomal RNA gene partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2 complete sequence and 28S ribosomal RNA gene partial sequence

The most potent acidophilic glutaminase producing isolate (F1.a) has been identified genetically based on 18S ribosomal RNA gene partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2 complete sequence and 28S ribosomal RNA gene partial sequence and has shown 93% similarity to *Aspergillus niger* strain CPGM 1439.

Table 5. Effect of different amino acids on enzyme activity for acidophilic isolate (F1.a)

Amino acid	Released NH_4^+ M	Enzyme activity (U/mL)
Alanine	0.04401	44.01
Glycine	0.03103	31.03
Glutamic acid	0.02948	29.48
Asparagine	0.03815	38.15
Aspartic	0.03492	34.92
Iso-Leucine	0.04959	49.59
Cysteine	0.03607	36.07
Valine	0.03518	35.18
Tyrosine	0.03581	35.81
Proline	0.00171	1.71
Control	0.03596	35.96

Among different sources of L-glutaminase producers, fungal strains possess an elevated edge over others due to their growth requirements and easy processing [50]. Reports suggested that these enzymes produced by different microorganisms differed in some physiological, biochemical, catalytic and immunological properties. This led to the continuous screening program for isolation of novel microbial strains that could produce an effective enzyme with few limitations at use.

Production and optimization for glutaminase enzyme by fungi was reported for many species as *Trichoderma reesei* [14], Genus *Aspergillus was on the top of producers list as Aspergillus flavus* [51], *Aspergillus* sp. [27] *Aspergillus fumigatus* WL002 [52], *Aspergillus wentii* MTCC 1901 [46] *A. niger*, *A. fumigatus*, *A. phoenicis* and *Penicillium funicolosum* was also reported as glutaminase producer [53].

4. CONCLUSION

Glutaminase enzyme is very important enzyme to be studied extensively and produced due to its applications in medical and industrial fields. This research aimed to isolate an efficient acidophilic glutaminase producing microorganism. *Aspergillus niger* CPGM 1439 was the most potent selected isolate and produced glutaminase enzyme with high yield of about 50 U/mL after studying seven environmental and five nutritional factors that can affect the enzyme productivity. Also, it is a great advantage that it was produced at very low acidic pH 1.8 suggests it has high prospect for industrial applications since stability in acidic pH is one of the properties of enzymes required for potential industrial applications. It is therefore; recommend further studies carried out for modeling the production and production on large scale fermenters which is expected to be completed in our ongoing studies. Also, it is suggested to try more natural or wastes as media for production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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