Pharmacophore

ISSN-2229-5402

Journal home page: http://www.pharmacophorejournal.com

GENETICS IDENTIFICATION AND DETECTION OF L-METHIONINASE GENE IN A *PSEUDOMONAS* ISOLATE

Al-Zahrani, SHM[1], Bukhari, KA[2*]

1. *Biology department, Faculty of Science, King Abdulaziz University, KSA*
2. *Biology department, Faculty of Science, University of Jeddah, KSA.*

ARTICLE INFO

Received:
04 Aug 2019
Received in revised form:
07 Dec 2019
Accepted:
14 Dec 2019
Available online:
28 Dec 2019

Keywords: L-methioninase, methionine, anti-tumour, anti-cancer, *Pseudomonas*, gene identification, gene detection

ABSTRACT

Cancer is a growing cause of death and morbidity worldwide. L-methioninase (EC 4.4.1.11) has a potential application against many types of cancers like glioblastoma, kidney, breast, lung, and colon cancer. The present study aimed to screen microbial production of L-methioninase from marine samples obtained from Jeddah coast In Saudi Arabia. Sixteen bacterial isolates were obtained from different marine samples. The bacterial isolate's ability for L-methioninase production was tested on a modified M9 L-methionine agar medium using phenol red as the pH indicator. Colonies with pink-red and yellow zone around were selected as L-methionine degrading bacteria. Results revealed the incubation of the cultures in a shaking incubator (150rpm) led to decreased L-methioninase production by some isolates but increased the creation of the enzyme by other isolates after 48 hours of incubation. The highest L-methioninase producing isolate (*Pseudomonas extremaustralis*) was obtained from a seashell, and identified based on 16*S* rDNA sequencing, along with the biochemical characterization, BLAST analysis, and the structure of a phylogenetic tree. The rRNA sequence of *P. extremaustralis* was deposited in GeneBank (NCBI accession number MK072729).

To Cite This Article: Al-Zahrani, SHM, Bukhari, KA, (2019), "Genetics Identification And Detection Of L-Methioninase Gene In A *Pseudomonas* Isolate", ***Pharmacophore, 10(6),*** *93-98*.

Introduction

L-methioninase (EC 4.4.1.11) is a pyridoxal phosphate (PLP) dependent hydrolytic enzyme and also known as L-methionine-γ-lyase, L-methionine-γ-demethylase, methioninase, and L-methionine-methanethiol-lyase. It is absent in the mammalian system and intracellularly present in bacteria and extracellularly in fungi [1]. This enzyme was first isolated from the rumen bacteria [2]. Disruption and abnormalities in methionine metabolism and transmethylation are associated with the primary disease in humans, like aging, obesity, Parkinson's disease, heart disease, and cancer. The therapeutic application of L-methioninase was studied from bacteria, yeast, and fungi. L-methioninase secreted from bacterial sources is mostly associated with high immunogenicity, low substrate specificity, and hazardous effects on the kidney and liver [3]. Normal and malignant cells may differ in their amino acid requirements [4, 5].

In consequence, dietary restrictions of amino acids [6-8] have been used successfully to treat-tumor-bearing animals [2]. L-methioninase has high therapeutic value since it was reported as a potent anticancer agent against various types of tumor cell lines like glioblastoma, kidney, breast, colon, and lung cancer [9, 10]. L-methionine is an essential amino acid that regulates cellular metabolism by synthesizing polyamines and glutathione which are further used for biosynthesis of protein and gene expression [11, 12]. The significant activity of this enzyme has been highly observed in patients of high-stage gastric cancer after applying chemotherapeutic drugs with methionine-free diet [13, 14]. Screening for new producers and new kinds of growth medium for the production of this enzyme with additional therapeutic characters to fulfill their requirements will be a challenge. Our work concentrated on screening for L-methioninase-producing bacteria isolated from the Saudi environment.

Corresponding Author: Bukhari, KA. Biology Department, Faculty of Science, University of Jeddah, KSA. Email: Kawtharbukhari @ hotmail.com

Materials and methods

Collection of samples

Different marine samples (solid coral, marine sponge, seashells, algae, and sea mushroom) were collected from the marine environment of the west coast of Jeddah city in Saudi Arabia, and the samples were placed in zip-locked plastic bags.

Isolation and qualitative estimation of L-methioninase producing bacteria by rapid plate method

Serial dilutions from different samples were screened for bacterial isolates have the ability of L-methioninase production by a qualitative rapid plate assay procedure using a modified M9 medium, $Na_2HPO_4.2H_2O$ 6.0, KH_2PO_4 3.0, NaCl 0.5, L-methionine 1, $MgSO4.7H_2O$ 0.25, $CaCl_2$ 0.014, Glucose 2, agar 20gm/ L., pH 7±0.2 and phenol red was added to the medium as an indicator at a final concentration of 0.009% just before pouring the plates. The plates were incubated in an inverted position at 35±2°C for 24 to 48 hours. All bacterial colonies producing L-methioninase were selected on the base of formation a pink-red and yellow zone around the colonies. Well, isolated colonies purified, and subcultures from each plate were selected for further studies [15].

Estimation of L-methioninase in submerged culture by bacterial isolate:

A loop full of a 24h culture of the selected bacterial strain was inoculated with 30ml of a sterile seed medium in a test tube and incubated at 35±2°C in a constant incubator for 24h. The modified M9 broth medium was used as the fermentation medium supplemented with L-methionine with and without glucose, and after inoculated, the bacterial cultures incubated in shaking incubator (150rpm) and others in a static condition at 35±2°C, L-methioninase in the free-cell filtrate was assayed after 72h of incubation.

Quantitative evaluation of L-methioninase by rapid plate method:

L-methioninase evaluation by agar well diffusion method of cell-free filtrate culture (CFF) on solid media in Petry dishes, some supplemented with L-methionine as a substrate. Solidify media in the plates, 7mm wells were punching using sterilized cork borer, 100µl of CFF was loaded in the wells, the plates kept in an upright position at 35±2°C for 48 hours and the size of the area was measured [16].

L-methioninase assay by Nesslerization method:

The activity of L-methioninase was determined by the Nesslerization method [17] with some modifications. The standard reaction system contains 1 ml of 1% L-methionine in 0.5M phosphate buffer (pH 7.0), 0.1ml of pyridoxal phosphate, and 1 ml of crude enzyme. The reaction system was incubated at 30°C for one hour. The enzymatic activity was stopped by adding 0.5ml of 1.5mol/ L Trichloroacetic acid. The system was centrifuged at 5,000rpm for 5min to remove the precipitated protein. 0.1 ml of above the mixture was added to 3.7ml of the distilled water, and the released ammonia was determined using 0.2ml of Nessler reagent, the developed colored compound was measured at 480nm using a spectrophotometer. Enzyme and substrate blanks were used as controls. One unit of L-methioninase was defined as the amount of enzyme that liberates ammonia at 1µmol/ min under optimal assay conditions. The specific activity of L-methioninase was expressed as the activity of the enzyme in terms of units per milligram of protein [18].

Identification of the most L-methioninase producing isolates *Pseudomonas* sp.:

The cultural characteristics, morphological, physiological, and biochemical properties were investigated. Different microbial identification tests were performed according to the Berge's Manual of Determinative Bacteriology.

PCR amplification of 16S rRNA gene and L-methioninase gene:

For L-methioninase, bacterial isolate 16S rDNA universal primers were ordered by [19]. The PCR reaction conditions follow initial denaturation at 94°C for 5min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min; followed by a final extension at 72°C for 10 min. Primers to amplify the L-methioninase gene of *Pseudomonas extremaustralis* strain were ordered according to *Pseudomonas extremorientalis* sequence of L-methioninase gene (NCBI accession number LT629708.1) ***Pe*-F** AGAGTTTGATCCTGGCTCAG and ***Pe*-R** GCAACGCGCATGATCTACTG. The reaction conditions follow Initial denaturation: 5min at 94°C, Denaturation: 1min at 94°C, Annealing: 1min at 55°C and Extension: 2min at 72°C. A number of cycles: 35. Final extension: 10 min at 72°C by a thermal cycler (Bibby Scientific, UK).

Results

Isolation and qualitative method for L-methioninase producing bacteria by rapid plate method:

Sixteen bacterial isolates were obtained from marine samples, six, three, four, two, and one bacterial strain were derived from the solid coral, marine sponge, seashells, algae, and sea mushroom samples, respectively. Bacterial isolates with the ability of L-methioninase production were tested on a modified M9 medium.

Quantitative evaluation of L-methioninase by rapid plate method:

The quantitative evaluation of L-methioninase was performed by agar well diffusion assay. Cell-free culture filtrate (CFF) was supplemented with L-methionine as the sole organic source, and another one supplemented with L-methionine as nitrogen source and glucose as carbon source. Results showed that there were differences in the diffusion of the CFF of the isolates in the agar depending on the isolates, the presence of glucose and methionine or only methionine as an organic source, as well as shaking and constant incubation.

The biggest diameter of the L-methioninase zone was observed in CFF supplemented with L-methionine as the sole organic source. It was 30mm and was produced by isolate S.s1 that isolated from a seashell, incubated in shaking incubator (150rpm) (Fig.1).

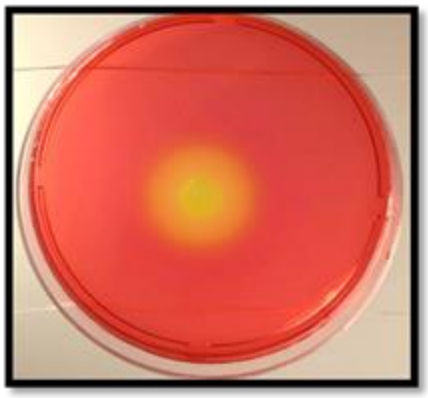

Figure 1: Quantitative estimation of L-methioninase production by rapid plate method and the pink-red and yellow color clearly showed the zone size.

Estimation of L-methioninase in submerged culture by bacterial isolates:

This experiment was conducted to study the effect of incubation of cultures in shaking incubator (150rpm) compared to constant incubator on the production of L-methioninase in cultures. L-methioninase amounts in CFF were estimated by Nessler assay. Results in figure (2) show that incubation in a shaking incubator (150rpm) led to decreased L-methioninase production by some isolates but increased the production of the enzyme by other strains after 48 hours of incubation at 35±2 °C. L-methioninase production was higher in the constant incubator by three bacterial isolates in culture supplemented with glucose as a carbon source, while incubation of five bacterial isolates in a shaking incubator maximized their productivity of L-methioninase. The highest L-methioninase production was by four bacterial isolates under constant incubation, and two bacterial isolates under the shaking incubation with L-methionine as the sole organic source. It was noticed that the highest L-methioninase production was in the shaking incubator in the presence of L-methionine with a carbon source. The production of L-methioninase by isolate S.s1 that isolated from the seashell sample under shaking incubator (0.036U/min/ml) was higher than that in the contestant incubator. The production was 0.0282U/min/ml in both conditions in the presence of glucose. This bacterial isolate was chosen for further studies.

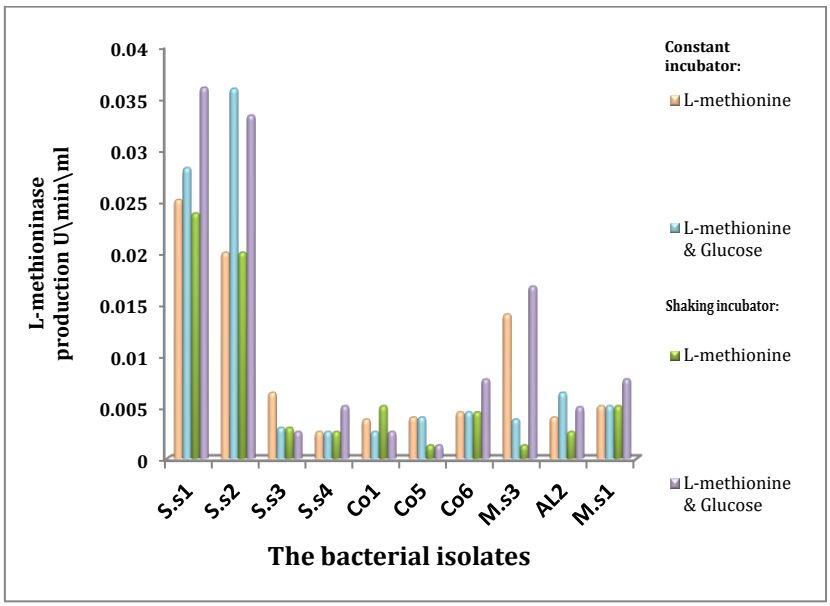

Figure 2: Effect of shaking incubation on L-methioninase production.

Phylogenetic analysis of *Pseudomonas extremaustralis* isolates based on 16S rDNA

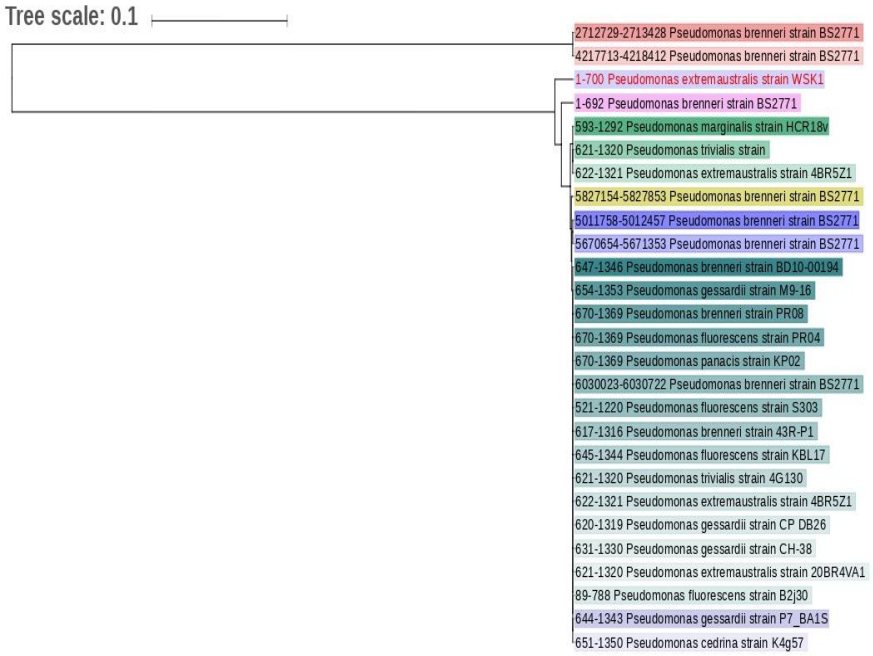

Figure 3: Neighbor-joining tree showing the phylogenetic position of *Pseudomonas extremaustralis* strain and their related species based on partial 16S rRNA gene sequences. The strain used in this study, WSk1, takes a red color.

Molecular detection of L-methioninase gene in *Pseudomonas extremaustralis* strain by PCR

The gel electrophoresis image shows the presence of L-methioninase gene in *Pseudomonas extremaustralis.* L-methioninase gene presence in *Pseudomonas extremaustralis* was confirmed with the expected PCR yield of 515bp (Figure 4).

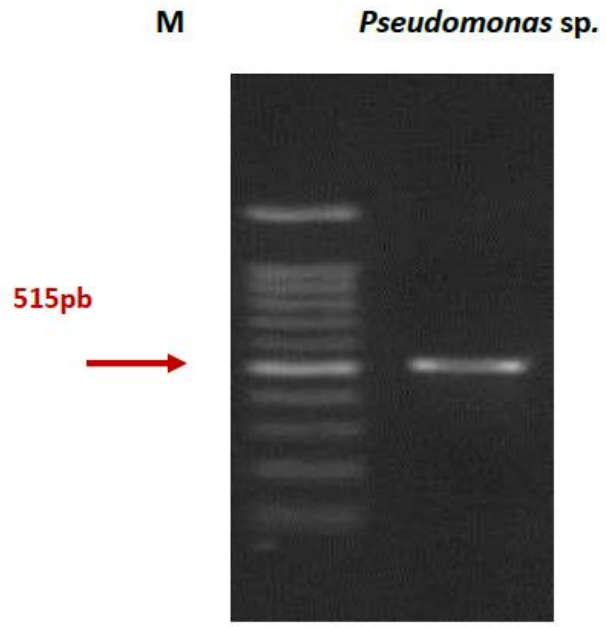

Figure 4: PCR amplification of the L-methioninase gene from *Pseudomonas extremaustralis* strain. Lane 1: 100bp ladder; Lane 2: 515bp partial L-methioninase gene.

Discussion:

Phenol red shows the fundamental pH change, which is red in alkaline conditions, turning to yellow under acidic conditions [20]. Similarly, this technique was used to study the dissimilation of L-methionine by L-methioninase. The observations

were recorded after 72 hours for the presence of pink-red and yellow zone formed due to the formation of ammonia and α-keto butyric acid. In case L-methionine is metabolized, a change in the color of the medium to pink-red and yellow would be observed due to the presence of red phenol indicator, which is pink-red in basic condition and turns yellow under acidic condition. Therefore, the isolates showing pink-red and yellow zone on a modified M9 medium for primary screening were selected. In this study decrease in pH may be due to the formation of α-keto butyric acid. Hence, pH change will lead to the change in color to yellow; this result agrees with [15]. While ammonia formation rises alkalinity of the L-methionine-agar medium, the color changes to pink-red; this result agreed with that found by [21-23]. The production of L-methioninase under submerged conditions has been reported by [24, 25]. The fermentation is a metabolic process in many microorganisms and involves oxide-reduction reactions resulting in the breakdown of complex organic compounds into simpler by-products and energy. The microbes carry out this metabolic action by the release of 27 extracellular enzymes [15]. Culture broths on L-methionine glucose media have shown good L-methioninase production under submerged conditions [18]. This media give the necessary minimal nutrition required and provides L-methionine as a carbon source, which is used by bacteria as a growth substrate.

This media allows the growth of even those bacteria which can use glucose, therefore, to further screen the obligate L-methionine degraders, 100% L-methionine basal agar was used, where L-methionine is the only sole source of carbon, nitrogen [26]. In cultures containing L-methionine as the nitrogen source, glucose as carbon source, and incubated in shaking incubator, seven bacterial isolates were found to produce L-methioninase. Thus, the strains that showed the activity in the CFF were considered potent producers. The acid production takes place in both cases; L-methionine utilization produces α-keto butyric acid, whereas glucose utilization produces organic acids. Therefore in this study, the result confirmed the presence of the enzyme when L-methionine was used as the sole organic source. The result also showed that the enzyme production was enhanced in the presence of carbon source maybe because the carbon source acts as growth-supporting; this result agrees with that found by [18, 21, 26, 27].

Sharma *et al*. (1991) revealed that this system offers numerous advantages, including high volumetric productivity, relatively higher concentration of the products, less affluent generation, and the requirement for simple fermentation equipment [28]. Shaking velocity is known as a possible parameter affecting enzyme productivity. Some isolates produced the highest amount of the enzyme in shaking cultures. This may be attributed to the mechanical forces that can lead to the vacillation of older hyphal compartments, which may lead to weakened hyphae and/or accelerating hyphal fragmentation, but in fungi, [29] demonstrated that, physiologically, SSF has enzyme manufacturing potential.

The DNA sequences were analyzed using Blast alignment tools of GenBank and the isolate was identified as *Pseudomonas extremaustralis* with 100% identity. The partial 16S rDNA sequence of the selected isolate was submitted into the Bacterial or Archaeal 16S ribosomal RNA sequences database under the accession numbers: MK072729 (*P. extremaustralis* strain WSk1 isolated from a seashell). L-methioninase gene was successfully isolated from *P. extremaustralis* strain. The L-methioninase gene has been amplified, giving the expected molecular weight of 515bp.

Conclusion

The present study reports the production of L-methioninase by bacterial isolate, which was isolated from seashell samples. The isolate was identified by molecular methods as *Pseudomonas extremaustralis*. To the best of our knowledge, this is the first report on the isolation and production of L-methioninase by *P. extremaustralis*, and the L-methioninase can be a potential candidate as an anti-tumor enzyme.

Acknowledgment

Thanks to the Biology Department, Faculty of Science, the University of Jeddah, for their cooperation and guidance.

References

1. Sharma B, Singh S, Kanwar SS. L-methionase: a therapeutic enzyme to treat malignancies. BioMed research international. 2014;2014.
2. Kallio R, Larson A. Methionine degradation by a species of Pseudomonas. Paper presented at: A symposium on amino acid metabolism. Johns Hopkins Press, Baltimore, 1955.
3. Sun X, Yang Z, Li S, et al. In vivo efficacy of recombinant methioninase is enhanced by the combination of polyethylene glycol conjugation and pyridoxal 5′-phosphate supplementation. Cancer research. 2003;63(23):8377-8383.
4. McCoy TA, Maxwell M, Kruse PF. The amino acid requirements of the Jensen sarcoma in vitro. Cancer Res. 1959;19(6 Pt 1):591-595.
5. Ohnuma T, Waligunda J, Holland JF. Amino acid requirements in vitro of human leukemic cells. Cancer research. 1971;31(11):1640-1644.

6. Faid SM, Al-Matrafi MM. Evaluation of Okra Pods Quality (Abelmoschus esculentus L.) After Reduction of Pesticides. J Biochem Tech. 2018;9(4):81-88.
7. Gull M, Kausar A. Essential Nutrients' Uptake Potential and Biochemical Characterization of Four Genotypes of Wheat (Triticum Aestivum L.) Under Various Cdcl2 Stress Conditions. J Biochem Tech. 2018;9(1):53-60.
8. Aaj Bishe N, Shokri A. The Use of Phosphates in the Meat Industry. J Biochem Tech. 2019;Special Issue (2):90-96.
9. Kokkinakis DM, Hoffman RM, Frenkel EP, et al. Synergy between methionine stress and chemotherapy in the treatment of brain tumor xenografts in athymic mice. Cancer research. 2001;61(10):4017-4023.
10. Tan Y, Sun X, Xu M, et al. Polyethylene glycol conjugation of recombinant methioninase for cancer therapy. Protein expression and purification. 1998;12(1):45-52.
11. Davis CD, Uthus EO. DNA methylation, cancer susceptibility, and nutrient interactions. Experimental biology and medicine. 2004;229(10):988-995.
12. Baylln SB, Herman JG, Graff JR, Vertino PM, Issa J-P. Alterations in DNA methylation: a fundamental aspect of neoplasia. Advances in cancer research. Vol 72: Elsevier; 1997:141-196.
13. Goseki N, Yamazaki S, Shimojyu K, et al. Synergistic effect of methionine-depleting total parenteral nutrition with 5-fluorouracil on human gastric cancer: a randomized, prospective clinical trial. Japanese journal of cancer research. 1995;86(5):484-489.
14. Guo H, Lishko VK, Herrera H, Groce A, Kubota T, Hoffman RM. Therapeutic tumor-specific cell cycle block induced by methionine starvation in vivo. Cancer research. 1993;53(23):5676-5679.
15. Bahl C, Saxena SG, Sharma SG. Screening endophytic fungal broth for l-Methioninase activity; 2012.
16. Jain, Richa, Zaidi, et al. L-asparaginase: A promising enzyme for treatment of acute lymphoblastic leukiemia. 2012.
17. Imada A, Igarasi S, Nakahama K, Isono M. Asparaginase and glutaminase activities of micro-organisms. Microbiology. 1973;76(1):85-99.
18. El-Sayed AS. L-methioninase production by Aspergillus flavipes under solid-state fermentation. Journal of basic microbiology. 2009;49(4):331-341.
19. Buonaurio R, Stravato V, Cappelli C. Brown spot caused by Sphingomonas sp. on yellow Spanish melon fruits in Spain. Plant pathology. 2001;50(3):397-401.
20. Theantana T, Hyde K, Lumyong S. Asparaginase production by endophytic fungi isolated from some Thai medicinal plants. KMITL Science and Technology Journal 2007;7.31:13-18.
21. Abu-Tahon MA, Isaac GS. Comparative study of a new alkaline L-methioninase production by Aspergillus ustus AUMC 10151 in submerged and solid-state fermentation. Brazilian Archives of Biology and Technology. 2016;59.
22. Selim M, Elshikh H, El-Hadedy D, Saad M, Eliwa E, Abdelraof M. L-Methioninase from some Streptomyces isolates I: Isolation, identification of best producers and some properties of the crude enzyme produced. Journal of Genetic Engineering and Biotechnology. 2015;13(2):129-137.
23. El-Sayed AS, Shindia AA. PLP-Dependent Enzymes: a Potent Therapeutic Approach for Cancer and Cardiovascular Diseases. Targets in Gene Therapy: InTech; 2011.
24. Raimbault M, Alazard D. Culture method to study fungal growth in solid fermentation. European Journal of Applied Microbiology and Biotechnology. 1980;9(3):199-209.
25. Khalaf SA, El-Sayed AS. L-Methioninase production by filamentous fungi: I-screening and optimization under submerged conditions. Current microbiology. 2009;58(3):219-226.
26. Ruiz-Herrera J, Starkey RL. Dissimilation of methionine by fungi. Journal of bacteriology. 1969;99(2):544-551.
27. Lockwood BC, Coombs GH. Purification and characterization of methionine γ-lyase from Trichomonas vaginalis. Biochemical Journal. 1991;279(3):675-682.
28. Sharma D, Niwas S, Behera B. Solid state fermentation of bagasse for the production of cellulase enzyme from cellulolytic fungi and extent of simultaneous production of reducing sugars in the fermenter. Journal of Microbial Biotechnology. 1991;6(1):7-14.
29. Paul GC, Kent C, Thomas C. Hyphal vocuolation and fragmentation inpenicillium chrysogenum. Biotechnology and bioengineering. 1994;44(5):655-660.