Educational Administration: Theory and Practice
2024, 30(6)(s), 44 - 49
ISSN: 2148-2403
https://kuey.net/

Research Article

Isolation, Screening And Morphological Identification Of Fungal L-Methionase From Rotten Fruits And Vegetables

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Citation: Anmol Kumar, et.al (2024) Isolation, Screening And Morphological Identification Of Fungal L-Methionase From Rotten Fruits And Vegetables, Educational *Administration: Theory And Practice, 30(6)(s), 44 - 49*
Doi: 10.53555/kuey.v30i6(S).5320

ARTICLE INFO

ABSTRACT

Globally, cancer is becoming a more common cause of both mortality and morbidity. L-Methionase is one of the few microbial enzymes with high therapeutic value since it has been reported as an effective anticancer agent against many types of tumor cell lines like breast, lung, colon, kidney, and glioblastoma. Normal cells have a high requirement of amino acids as nutrients, without amino acids, tumor cells fail to function because protein can't be synthesized and tumor cells will die. L-Methionase has important biotechnological applications because of exhibits hydrolytic properties to catalyze α-γ elimination of L-methionine, an essential amino acid to α-ketobutyrate, methanethiol, and ammonia. The catalytic activity of L-Methionase could be used as enzyme supplementation therapy for these diseases. enzyme has been extensively studied by a wide range of organisms, including bacteria, fungi, protozoa, and plants. As a result, of the current study, we obtain 24 fungal isolates from rotten fruits and vegetables. Qualitative and quantitative screening assay test shows the ability of seven isolates to grow with yellow zones surrounding their colonial
growth. MFL 9 (3.45 µmol/min/ml) and MFL24 (2.95 µmol/min/ml) exhibit the highest L-Methionase enzyme activity according to the Nesslerization assay performed for the detection of ammonia. Therefore, the current study indicates that the enzyme extract from the new isolates has enhanced L-Methionase activity and could be a novel and promising therapy for cancer.

Keywords: Anticancer Enzyme; L-Methionase; Therapeutic Potential; Fungi

INTRODUCTION

Nowadays, anticancer drugs are either monoclonal antibodies or low-weight substances that block the critical phase of cancer growth to prevent it from proliferating. Cancer prevention and treatment are difficult challenges [1]. Cancer cannot be completely prevented, and current therapies may have adverse effects. This article highlights current efforts to synthesize L-Methionase, an enzyme that some fungi use as a natural defense mechanism that helps the human body fight off diseases, including breast cancer, lung cancer, kidney cancer, colon cancer, glioblastoma and renal cancer [2].

The use of enzyme therapies in medicine growing as more manufacturers focus on their benefits in treating disease. Enzymes are unique among pharmaceuticals for two main reasons. Firstly, enzymes usually bind to their specified substrates and function on them with high specificity and affinity. Second, they are catalytic so Enzymes may convert several target molecules into the required end products [3]. Enzymes are powerful medications that, unlike small molecules, may affect the human body's therapeutic biochemistry. These two essential features make enzymes distinct and potent. As a result, different enzyme-based medicines have been developed to treat various medical conditions [4].

Research indicates that bacterial enzymes can harm the kidneys and liver as well as become highly immunogenic and have poor substrate selectivity. Fungal-derived L-Methionase is an effective enzyme source for anticancer therapy. Since it has a high substrate selectivity and a low immunogenic and allergic reaction[5].

L-Methionase is present in all living organisms including, bacteria, molds, protozoa, and plants except mammals[6]. L-Methionase is a pyridoxal phosphate (PLP) dependent hydrolytic enzyme also commonly referred to as L-methionine-γ-lyase, L-methionine-γ-demethiolase, L-Methionase, and L-methionine-methanethiol-lyase[7]. The enzyme directly converts L-methionine into α-ketobutyrate, ammonia, and methanethiol[8]. L-Methionase is One of the rare microbial enzymes with great therapeutic efficacy. It is a potent antitumor agent that works against a variety of cancer cell lines, including those derived from colon, breast, lung, kidney, and glioblastoma[9]. L-methionine is an important amino acid that is required for the proliferation and formation of many different primary tumors, including human carcinoma cells. On the other hand, in active Methionase synthase, normal cells can grow on homocysteine rather than L-Methionase[10]. Consequently, this work aims to determine isolation, screening, and morphological identification of anticancer characteristics of L-Methionase from fungi isolated from rotten fruits and vegetables.

MATERIALS AND METHODS

A. Materials

The chemicals used in this study include potato dextrose agar, C'zapex dox agar, phenol red, L-Methionine, Nessler's reagent, trichloro acetic acid, potassium phosphate buffer, and Pyridoxal-5-phosphate all chemical purchased from Himedia, Maharashtra, India.

B. Collection of samples

Rotten fruits, as well as vegetables such as pineapple, banana, orange, apple, kiwi, tomato, onion, and potatoes, were collected in sterile polythene bags from the local market of Rajkot. The samples were later transported to the laboratory. Further, studies were done to isolate and identify the fungus from the above samples.

C. Isolation of Fungi

Isolation of fungi from rotten fruits and vegetables was collected from the local market at Rajkot. Some rotten portions were cut with a sterile scalpel and surface sterilized with 1% hypochlorite for 2 min and rinsed in several changes of distilled water. The thin section was put into Potato Dextrose Agar (PDA), containing Potato infusion (4g/l), Dextrose (20g/l), and Agar-Agar (20g/l) medium amended with streptomycin The plate was incubated at 28 ℃ for 7days. The fungal isolates were sub-cultured and maintained on the same media**.** A pure culture was obtained and maintained by subculturing each of the different colonies that emerged onto the PDA plates and incubating at 28 ℃ for 7 days.

D. Qualitative Screening Assay of L-Methionase

Using a qualitative screening plate assay method with C'zapek Dox agar containing sucrose (30g/l), sodium nitrate (2.0 g/l), dipotassium phosphate (1.0 g/l), magnesium sulfate (0.5g/l), potassium chloride (0.5g/l), ferrous sulfate (0.01g/l) and agar (15 g/l) medium amended with L- methionine amino acid used as carbon source and phenol red with final concentration of 0.007% just before pouring the plates, phenol red as an indicator was added to the media. The plates were incubated in an inverted position at 28℃ for 3 to 5 days. The choice of producing colonies depended on the formation of a yellow color zone surrounding the colonies containing L-Methionase. From each plate, well-isolated colonies that have been purified were selected for further investigation.

E. Quantitative Screening Assay of L-Methionase

In the Quantitative Screening Assay method fungal mycelium was inoculated into C'zapex broth. After inoculation broth was incubated in a rotary shaker at 120 rpm for 3 to 5 days. After incubation, fungal cell-free filtrate was obtained by centrifugation at 6000 rpm for 30 min. C'zapex Dox Agar plates supplemented with Phenol red and L-methionine are incorporated before pouring the plates. Plates were solidified 7mm wells were punched, using a sterile cork borer, 100µl of the fungal cell-free filtrate was loaded in the wells, and the plates were kept in an upright position at 28℃ for 24 to 48 hrs. After incubation, the formation of the yellow-colored zone around the well against a red background, the zone of diameter (mm) of L-Methionase, was measured.

F. Nesslerization Enzyme Assay for L-Methionase

The L-Methionase production was detected by determining the amount of ammonia released from L-methionine. The optimal reaction system includes 1 ml of 1% methionine in 0.5 M potassium phosphate buffer (PH 7.0), 0.1 ml pyridoxal phosphate, and 1 ml of raw enzyme. The reaction system was incubated at 30℃ for 1 h. The enzymatic activity was blocked by adding 0.5 ml of 1,5 mol/L trichloroacetic acid or by boiling for 5 min. The system was centrifuged at 5,000rpm for 5 min to eliminate the precipitated proteins. 0.1 ml prior mixture was added to 3.7 ml of distilled water and the liberated ammonia was detected utilizing 0.2 ml Nessler reagent, and the developed color compound was measured at 480 nm using a spectrophotometer. Blanks for the substrate and enzyme were used as controls. One unit of L-Methionase was measured as the amount of enzyme that releases ammonia at 1µmol/min under standard examination conditions[11].

G. Morphological identification

Identification was done macroscopically and microscopically. For macroscopic identification, colony characteristics such as appearance, change in medium color, and growth rate were observed on petri plates. For microscopic identification, a thin smear of fungus isolates from 7 days 7-day-old culture was inoculated aseptically on a sterile glass slide using a sterile needle. A drop of lactophenol cotton blue was added and the mixture was covered with a clean cover slip and a slide was observed under the 40X objective lens of the light microscope.

RESULT

A. Isolation of fungi

Based on fungal isolation we obtained 24 fungal isolates from different rotten fruits and vegetables from the local market at Rajkot.

B. Qualitative Screening Assay of L-Methionase

L-Methionase activity was tested on C'zapex dox agar supplemented with L-methionine, as the nitrogen source and add phenol red as the pH indicator. The existence of a yellow color region around the colonies on C'zapex dox agar plates with L-methionine due to the liberation of the enzyme.

C. Quantitative Screening Assay of L-Methionase

The cell-free filtrate of two selected fungi was evaluated L-Methionase production by agar well diffusion assay on a C'zapex dox plate. Results in (Table 1&2) show the maximum amount of L-Methionase was produced by (MFL 24) and (MFL 9), the diameter of isolate number MFL 24 is (15mm) after 24 h and (20mm) after 48 and isolate number MFL 9 is (25mm) after 24 h and (35mm)

D. Nesslerization Assay of L-Methionase

L-Methionase production by the selected fungal isolates was assayed by Nessler's method. The result in (Figure 2) shows fungal isolate MFL 24 which produces (2.95 µmol/min/ml) and isolate MFL 9 which produces (3.45 µmol/min/ml)

E. Morphological Characterization

Morphological identification was carried out by observing macroscopic and microscopic character and revealed that two fungi have L-Methionase activity. Isolate MFL 9 colony grows slowly with colony diameter 1.5-2.5 cm with black color on PDA agar. This type has semi-rounded colonies with round conidia. Isolate MFL 24 colony diameter 0.5-1 cm, colonies were white like velvet and grew like cotton in the middle. Colonies growth was semi-round with semi-round conidia and branch conidiophores.

IV. DISCUSSION

The disease of cancer requires new approaches for therapy, as it efficiently evades all existing modes of treatments with more and more people succumbing to it. Hence current studies focus on different forms of chemotherapy that harness the unique properties of cancer cells such as methionine dependence. In our previous study, we were able to find a novel source of L-Methionase, the fungi, *Aspergillus Fumigatus* which demonstrated effective anticancer properties. Other bacteria reported to produce L-Methionase include *Citrobacter freundii*[12], *Brevibacterium lines*[13], *Clostridium sporogenes*[14], *Streptomyces sp*[15][11]. The aim of this study of isolate, screen, and identify L-Methionase from rotten fruits and vegetables by promising isolate from anticancer application. We focused this study on higher L-Methionase activity producing fungi.
Using L-methionine glucose medium, *Aspergillus flavipes* [9][16][17] has demonstrated efficient L-Methionase synthesis in submerged conditions. For the quantitative assessment of the fungal culture filtrate, the agar well diffusion technique can be used. Potent producers are defined as isolates that demonstrate activity on L-Methionase agar plates. Several investigations have been conducted in the last few years to enhance the plate assay technique, which uses different pH indicator dyes for screening for isolates that produce L-Methionase [18]. The plate assay method was chosen as a quick, easy, and semi-quantitative screening technique for the manufacture of L-Methionase. The basic pH change is shown by phenol red, which turns red in alkaline and yellow in acidic conditions. The dissimilation of L-methionine by L-Methionase was also investigated using this technique. Following 24 hours, observations were conducted to determine if the appearance of a yellow zone was a result of ammonia production caused by the phenol red indicator[19].
The study investigated the identification of methionine-degrading microorganisms, including fungi, yeast, and bacteria, by supplementing the culture medium with L-methionine as the only source of nitrogen. None of the nitrogen sources could create L-Methionase in the absence of L-methionine. It has also been reported that *Aspergillus flavipes* produce L-Methionase, which is dependent on L-methionine. In *Hafnia alvei*[20], the combination of L-methionine and L-asparagine produced the greatest number of enzymes. *H. alvei* showed independence from L-methionine in terms of growth medium, which is comparable to observations in other species like *Pseudomonas putida*[21] and *Geotrichum candidum*[22], even if the presence of L-methionine

increased the production of L-Methionase. Microorganisms depend on carbon sources in the culture medium for growth and metabolite synthesis. Depending on the microorganisms under study, different carbon sources have varied effects on the generation of L-Methionase. A suitable carbon source for *Aspergillus flavipes* to produce L-Methionase [26]. *Geotrichum candidum*, a cheese-ripening yeast, uses glucose as its preferred carbon source to produce L-Methionase, as reported by[23].

V. CONCLUSION

The unique catalytic reaction of L-Methionase and its limited distribution in pathogens but not in humans make this enzyme a promising target for designing novel chemotherapeutic agents. Tumor cells have higher methionine requirements as compared to normal cells. The higher requirement of methionine amino acid by rapidly growing tumor cells supports higher protein synthesis, yet it can be exploited by the use of methionine-based therapy to rapidly deplete the cancerous cells. As a result, in this study, we underwent isolation, screening, and morphological identification of fungal isolates from various rotten fruits and vegetables for L-Methionase production. We have observed seven isolates that showed yellow-colored zone surrounding their colonial growth. Isolates MFL 9 (3.45 µmol/min/ml) and MFL24 (2.95 µmol/min/ml) exhibited the highest L-Methionase enzyme activity according to the Nesslerization assay performed for the detection of ammonia. Enzyme extract of these two isolates indicated higher L-Methionase activity, thus this study can prove to be significant and might be a novel concept and promising treatment for cancer.

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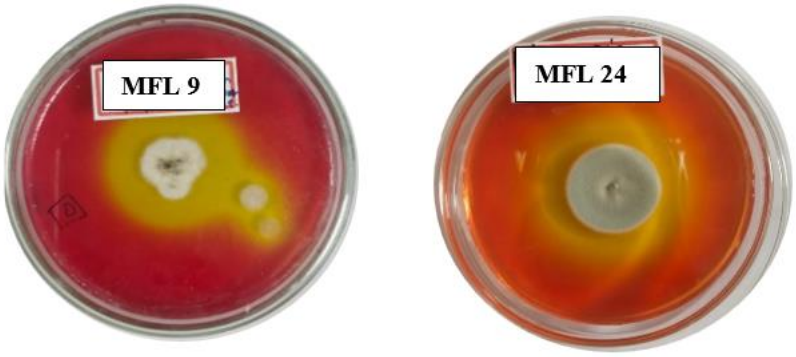
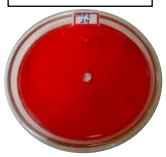


Fig. 1. Qualitative assay of L-Methionase with yellow color zone isolate number (MFL 24) and (MFL 9)

Table.1. Quantitative assays of L-Methionase in fungal cell-free filtrate by agar well diffusion method

Fungal Isolates	Control plate  **24hours**	**48 hours**
MFL 9		

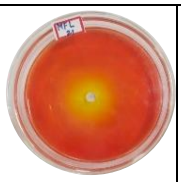
MFL 24		

Table. 2 Zone of diameter of MFL 9 and MFL 24

Fungal Isolates	**Zone of diameter in mm after**	**Zone of diameter in mm after**
	24 hr.	**48 hr.**
MFL 9	25	35
MFL 24	15	20

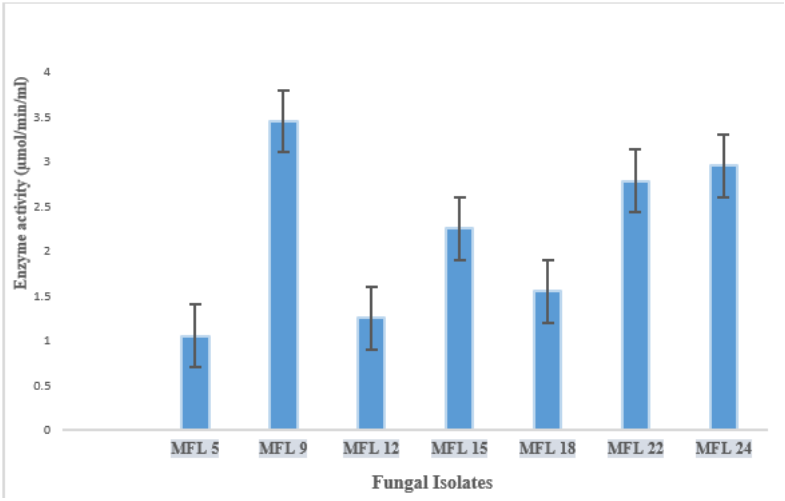


Fig. 2. L-Methionase activity produce by the selected fungal isolates by Nessler's Assay

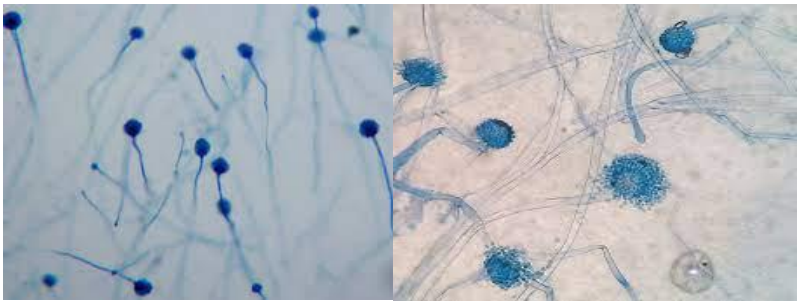


Fig. 3. Micro Morphological identification of fungal isolates