International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.9, No.4, pp 233-241, 2016

Production and optimization of L-glutaminase from a terrestrial fungal *Fusarium oxysporum*

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Abstract : L-glutaminase produced by adiverse group of microorganisms including fungi. L-glutaminase is presently used in the treatment of leukemia, HIVand also as flavor enhancing agent in food industries. Although L-glutaminase activity was reported in mostly of microorganisms, in our current research, L-glutaminase production modality was scrutinized under submerged fermentation using *Fusarium oxysporum* as anovel terrestrial fungal isolate which isolated from Egyptian soil using rapid plate assay procedure, then identified using different morphological and microscopic features. The maximum yield of enzyme production (2777 U/ml) was achieved at pH 6, 35°C, and 0.025% glutamine concentration after 7 days. The medium was inoculated with 100 µml / 30 ml of used medium supplemented with 1 % sucrose as carbon source.
Keywords: L-glutaminase;*Fusarium oxysporum*; submerged fermentation; enzyme optimization.

Introduction

Nowadays, medical biotechnology science illustrates the participation of glutaminase and other amino acid-depleting enzymes as agents for cancer therapy[1]. The outstanding challenge of cancer therapy was survived of cancer cells which can be plugged due to the mode action of L-glutaminase and the mostly auspicious usage of L-glutaminase is applied in the therapy of human immunodeficiency virus (HIV)[2]. L-glutaminase can be used as biosensors to control L-glutamine level in mammalian and hybridoma cell lines[3,4].L-glutaminase (EC 3.5.1.2), an amidohydrolase enzyme which formed L-glutamic acid and released ammonia from L-glutamine and it has an important role in cellular nitrogen metabolism[5].The production of primary and secondary metabolites and also enzymes basically rely on the genetic nature of the organism, constituents of growth medium and their concentration, surroundings growth conditions and interaction between all the above factors. Thus optimization of the previous conditions is necessary to get huge production and to improvement efficacious methods for industrial application. Many researchers informed that the optimization of physical and chemical conditions of growth medium could magnify microbial enzymes output[5,6].Most of microorganisms, involved bacteria, yeast, moulds and filamentous fungi have been informed to manufacture L-glutaminase[7-9].Glutaminlytic ability was informed in many terrestrial microorganisms such as *Escherichia coli, Pseudomonas sp, Acinetobacter sp, Bacillus sp, Proteus morganni, Candida* and *Aspergillus oryzea*[10]andalso few marine microorganisms havethe ability of L-glutaminase production as *Pseudomonas flurosence, Micrococcus luteus,* and *Beuveria bassiana*[11]. The most potent producers are fungi[12]. Although L-glutaminase can be synthesized from both plant and animal sources, microbial source is mostly used for industrial application because of their economic production, constancy, facility of modulation and optimization ways. In industry, glutaminases are

manufacture basically by *Aspergillus* and *Trichoderma*sp.[13-15]. The huge application of L-glutaminase in many areas always encouraged a search for a novel and economic source of the enzyme. Thus, the aim of this research, was the production of L-glutaminase from a potential and novel isolate *Fusarium oxysporum*under submerged fermentation and optimization of the growth parameters and nutritional factors of fermentation for consolidate the production of enzyme.

Material and methods

Microorganism, culture medium and maintenance conditions

Fusarium oxysporum used in this study was isolated from Egyptian soil, The fungal isolates were screened for their L-glutaminase production ability by qualitative rapid plate assay using modified medium, contains glutamine (5 g/l), glucose (10 g/l), KH_2PO_4 (1 g/l), K_2HPO_4 (1 g/l), $MgCl_2.6H_2O$ (0.5 g/l), $CaCl_2.2H_2O$ (0.1 g/l), $FeCl_3.6H_2O$ (0.02 g/l) and $ZnSO_4.7H_2O$ (0.02 g/l) all dissolved in 1 L of distilled water. The FinalpH of the medium was adjusted to 7.0, phenol red was added to the medium as indicator at final concentration of 0.007% just before pouring the plate and the plate incubated at 28°C for 7days, control plates of modified Czapek Dox's medium were (i) without dye and (ii) without glutamine[16]. The plates were then inoculated with 96 h cultures of tested isolates for rapid screening of glutaminase. The diameter of the colonies and the diameter of total clear hydrolytic halos including the colonies were determined. The strains that yielded higher halos were selected as potential microorganisms for L-glutaminase production using glutamine as substrate.

Rapid Confirmation of L-glutaminase by TLC method

Primarily screened strains were subjected to thin layer chromatography (TLC) for the confirmation of L-glutaminase production. Here the separation and identification of amino acids were carried out by thin layer chromatography technique by using silica gel G and saturated phenol with water used as a solvent system. The enzyme activity was estimated by redness of the spot developed by spraying ninhydrin reagent[17].

Morphological identification of L-glutaminase producer fungi

Identification of the isolated fungi during our investigation was carried out using the morphological characteristics as colony diameter, the color of condia, extracellular exudates, pigmentation and the color of reverse mycelium and microscopic features were examined also as conidial heads, fruiting bodies, degree of sporulation and the homogeneity characters of conidiogenous cells by optical light microscope (10×90) Olympus CH40 according to the following references: Ainsworth[18]and Booth[19,20]**.** Fungal isolates were grown onto Czapek Dox's medium at 28°C for several days (7-10). The cultures were then kept in 4°C.

Production of L-glutaminase

L-glutaminase producing isolate was taken up for studies of enzyme production in the same medium. The conidial suspension was prepared by injecting 10 ml of sterilized saline solution (0.85%) into a 7day old slant of selected fungus. 1 ml of spore suspension inoculating in 30 ml of modified liquid Czapek-Dox medium containing glutaminase in 100- ml Erlenmeyer conical flasks. The submerged cultures were incubated at 28°C for 7 days at 120 rpm.

L-glutaminase assay

L-glutaminase activity was assayed by direct Nesslerization according to the method of Thompson and Morrison[21]with some modifications. The standard reaction system contains 1 ml of 1% L-glutaminase in phosphate buffer (pH 7.0) and 1 ml of crude enzyme. The reaction system was incubated at 30°C for 1 h. The enzymatic activity was stopped by adding 0.5 ml of 1.5 mol/l trichloroacetic acid or by boiling for 5 minutes. The system was centrifuged at 5,000 rpm for 5 min to remove the precipitated protein. 0.1 ml of above mixture was added to 3.7 ml of distilled water and the released ammonia was determined using 0.2 ml of Nessler reagent, and the developed colored compound was measured at 480 nm using UV/VIS-2401 PC visible spectrophotometer (Shimadzu, Kyoto, Japan). Enzyme and substrate blanks were used as controls. One unit of L-glutaminase was defined as the amount of enzyme that liberates ammonia at 1 µmol/h under optimal assay

conditions. The specific activity of L-glutaminase was expressed as the activity of enzyme in terms of units per milligram of protein.

Determination of Extracellular Protein

The protein concentration of the crude enzyme preparation was estimated by Bradford reagent according to Bradford[22].

Biomass Determination

After the fermentation process, the cultures were centrifuged at 5,000 rpm for 10 min at 4°C followed by filtration through Whatman no. 1 filter paper. The cell pellets were washed with distilled water and dried at 80°C until a constant weight was achieved. The dry biomass was expressed as grams per liter of fermentation medium.

Effect of different parameters on L-glutaminase production

The impact of different parameters influencing L-glutaminase synthesis by *Fusarium oxysporum* was studied. The effect of different parameters on enzyme production was determined by incubating at different pH (6 to9 adjusted with 1 N HCl or 1 N NaOH), temperature (25, 30, 35 and 40°C), Incubation period (3, 5, 7and 9 days), inoculums size(25, 50, 100and 200µml), carbon sources (sucrose, glucose, maltose and starch at 1% w/v) and different glutamine concentration (2%, 4%, 0.5% and 0.025%),

Results and Discussion

Fusarium oxysporum was identified as glutaminlytic fungus as manifested by the pink color of fungal growth on the medium (Fig. 1). Generating from released of ammonia by the action of L- glutaminase on L-glutamine.

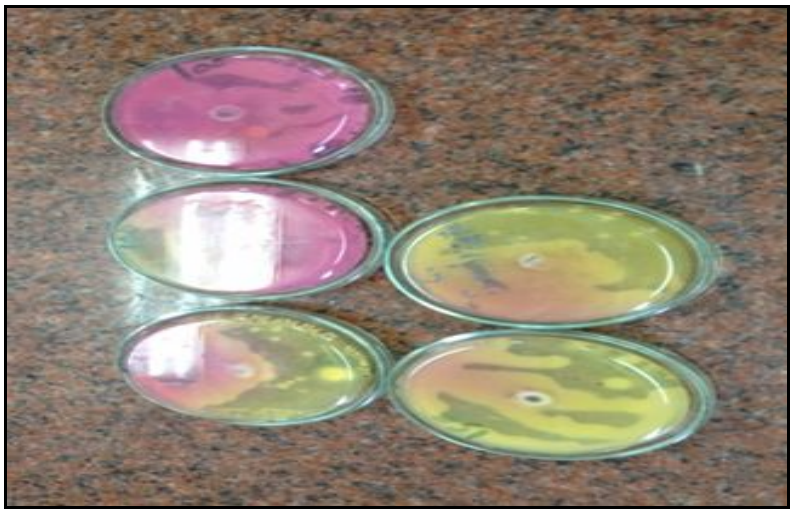

Figure 1. Rapid plate assay for L-glutaminase ability of *Fusarium oxysporum*.

Confirmation result of L-glutaminase production from *Fusarium oxysporum*using TLC are showed in Figure(2), The TLC technique was used for the separation and identification of L-glutaminase produced by *Fusarium oxysporum* which characterized by red spot developed after spraying ninhydrin reagent due to production of glutamic acid after the hydrolysis of glutamine by L-glutaminase enzyme which informed by the *Fusarium oxysporum*. In this research, the substance was produced by *Fusarium oxysporum* have similar Rf values (0.268) as that of standard glutamic acid (0.27).These results were comparable to that detected with filamentous fungi[23].According to available data, this is the former effort to assert L-glutaminase production by *Fusarium oxysporum*.

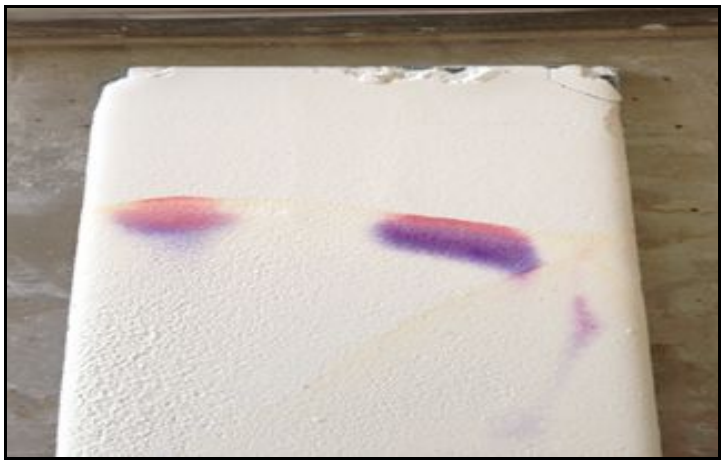

Figure 2: TLCconfirmation of L-glutaminase production by *Fusarium oxysporum.*

Morphological identification of *Fusarium oxysporum*.

The colony diameter of *Fusarium oxysporum*was4.2 cm after four days of incubation. The mycelia of *Fusarium oxysporum* was delicate, white to creamy and admix withpink to purple, margins slightly lobed or smooth. Microconidia formed singly, oval to reniform and without any septation. Conidiogenous cells bearing micro- and macroconidia were monophialides type. The size of microconidia ranged from 5.50 - 10.05 and 1.54 - 3.15 µm. Macroconidia were falcate to almost straight, usually 3-septed, rarely 5-septed, thin walled, both ends almost pointed, notched basal cell, apical cell short and in some cases slightly curved. Macroconidia were produced in sporodochia as well as on normal hyphae and slimy conidial masses were also observed on water soaked wheat bran. The size of the macroconidia ranged from 20.27 - 40.50 and 5.00 - 6.75 µm. Chlamydospores were thick walled, terminal or intercalary, globose, smooth or wrinkled, generally single celled (5.25 - 8.25 µm) produced in hyphae and conidia. Chlamydospores were also found in two celled or in cluster (9.5 - 30.0/7.5 µm) and in chain.

Effect of different parameters on L-glutaminase production

The microbial production of the enzyme depends on the genetic nature of the organism, the physiochemical parameters, the fermentation medium components and their concentrations. Hence, optimization of the above conditions is important to get maximal yields and to develop effective bioprocess system for industrial application. Many authors reported increased enzymes yield upon optimization of bioprocess conditions[9,24,25]. Optimization was achieved on “one parameter a time basis” i.e., by altering one distinct changeful while fixing the others at a specific stable level. The optimum conditions gained in each parameter was used to the further tests.

Effect of pH on the medium

The pH of the fermentation medium is informed to effect the growth and the physiology of microorganism. Generally, L-glutaminase production by most of the microorganisms under submerged fermentation conditions was noticed to be maximum production at pH 5.0 to 9.0. The results in Figure (3) mentioned that pH of the fermentation medium affected the enzyme production. Thus maximal enzyme output was noticed at pH 6.0 (2777.7 U/ml) with biomass (0.0117 g/l), either excess or lowering in the pH of the medium led to reduction of enzyme production. This may be referred to the equilibrium of ionic strength of plasma membrane. These results were in agreement with that informed by Sabu[26]but in our study the maximal enzyme output was higher than other available scientific reports

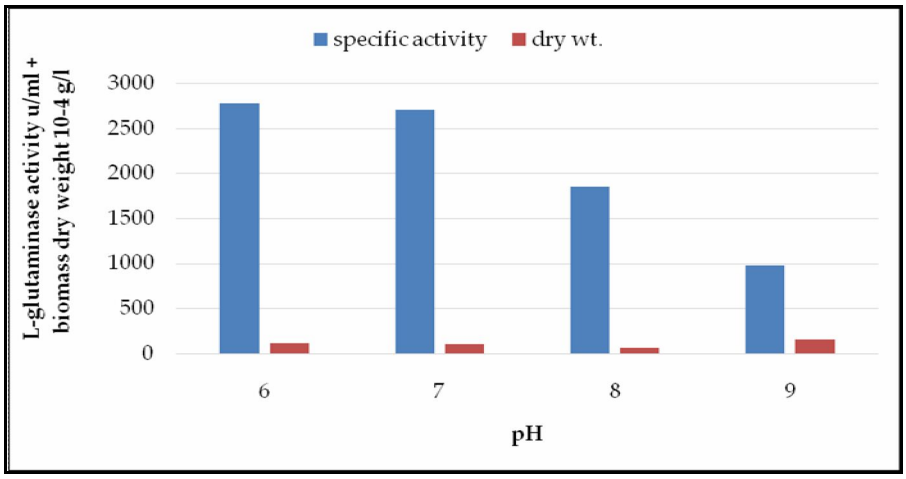

Figure 3. Effect of pH on L-glutaminase production.

Effect of temperature

Incubation temperature affected on levels of L-glutaminase production by *Fusarium oxysporum*, thus the maximal enzyme production (605 U/ml) with biomass (0.02675 g/l) was noticed at 35°C. Diversity in temperature in any way leads to reduce L-glutaminase production (Figure 4). These results were in harmonization with those informed by Kashyap[7] and Prasanna and Raju[27]. According to available data, the optimum temperature for L-glutaminase production is diversified with microorganism used. It was noticed that 27°C was optimum for the enzyme output by *Beauveria*sp. BTMP S10[28].

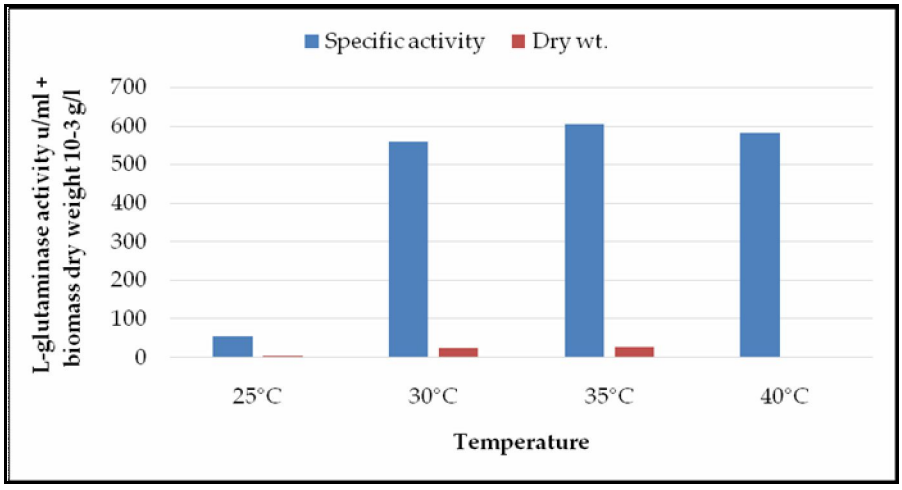

Figure 4. Effect of temperature on L-glutaminase production.

Effect of incubation period

Incubation time test was proceeded to control the level of L-glutaminase production by *Fusarium oxysporum*. Results in Figure(5) showed that, L-glutaminase production increase gradually until 7 days, when maximal enzyme production (49.33 U/ml) was recorded with biomass (0.0115 g/l). Enzyme activity reduced after 7 days and this may be due to existence of several types of proteolytic activity or the growth of the organism could have accomplished a stage from which it might no longer balance its steady growth with the availableness of nutrient sources which leading to inhibitory effect on enzyme, while in other literature reports, where it is noticed that the optimum incubation period for terrestrial fungi as *Aspergillus oryzae* were within 2 days[7,27].

Effect of inoculums size

The initial inoculums size controls the kinetics of growth and several metabolic functions leading to overall biomass and extracellular product formation[29]. To evaluate the same, experiments were planned with increasing inoculums concentration from 25, 50, 100, 150 and 200 µml. The results (Figure 6) indicated that the kinetics of L-glutaminase production varied with variation in inoculums concentration. The maximal enzyme production (1561.5 U/ml) with biomass was (0.0228g/ml) observed at 100µmlinitial inoculums supplementation and any other change in inoculums size leading to decrease in enzyme production and this result was in agreement with scientific report which was informed that the optimum inoculums size was recorded high enzyme production was100 µml[30].

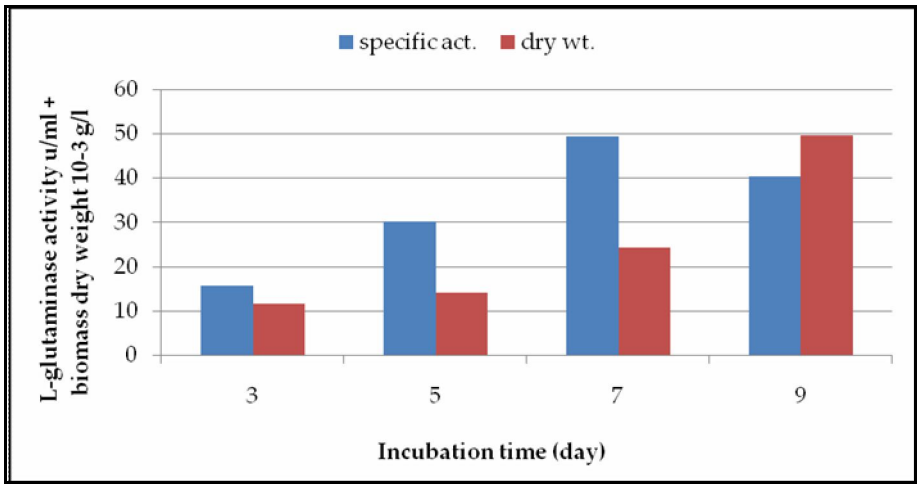

Figure 5. Effect of incubation time on L-glutaminase production.

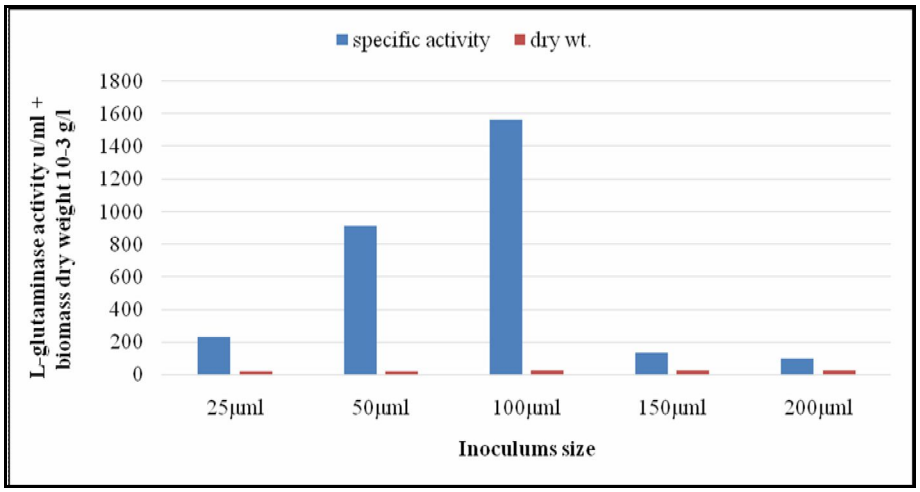

Figure 6. Effect of inoculumssize on L-glutaminase production.

Effect of Glutamine concentration

Need for glutamine as inducer substance for magnified L-glutaminase production was estimated by integrating various concentration of glutamine in the enzyme production medium. According to results in Figure (7), it was clear that, 0.025% concentration promoted maximal enzyme production (339.62 U/ml) with biomass (0.0094 g/l). With further increase in L-glutamine concentration, there was a gradual reduction in enzyme production which may be due to the inhibitory effect of L-glutamine at higher concentrations on the enzyme output. The same results were informed by Prasanth[31]but our results indicate that maximal enzyme production with very low L-glutamine concentration comparing with other researches and this make our tested isolate very economic L-glutaminase producer.

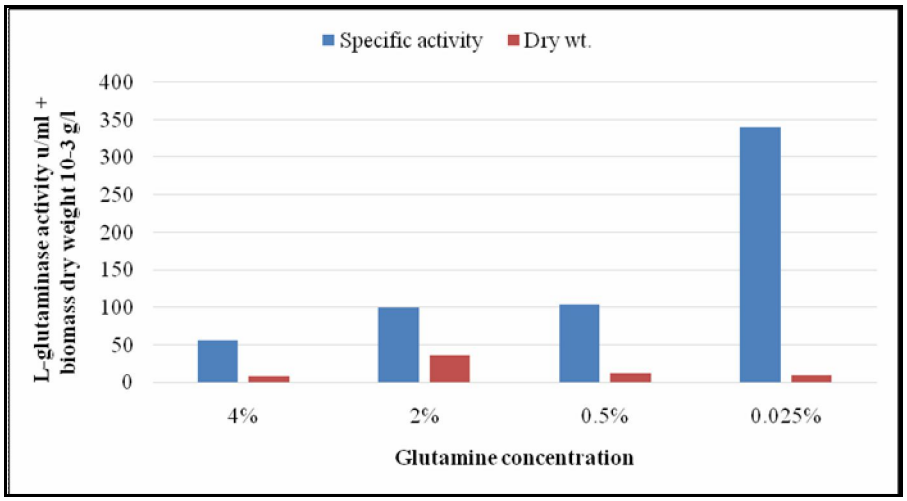

Figure7. Effect of different glutamine concentration on L-glutaminase production.

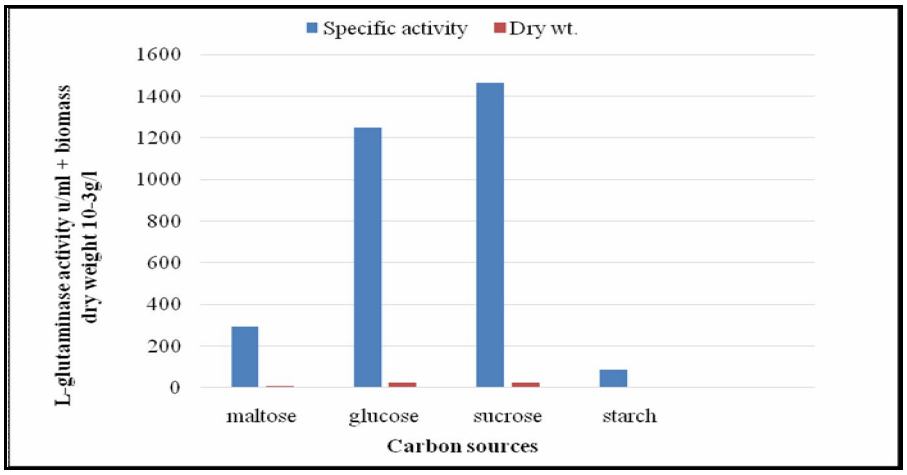

Figure 8. Effect of different carbon sources on L-glutaminase production.

Effect of carbon sources

Effect of carbon sources into medium at 1% level resulted in a respectable increase in the enzyme production. From all the diverse carbon sources tested, sucrose was supported maximal enzyme output (1465.44 U/ml), with biomass (0.0255 g/l). All the other carbon sources also showed considerable amount of enzyme production. Starch was the least effective as a carbon source (88 U/ml). From the result, it was noticed that all the other carbon sources except starch (0.0061 g/l) supported biomass production (Fig. 8).

Conclusion

The ability of *Fusarium oxysporum* which isolated from Egyptian soil for L-glutaminase production was tested under submerged fermentation with diverse process parameters and medium constituents. Maximum production was noticed at pH 6, 35°C, 0.025% glutamine concentration, sucrose as a sole carbon source, 7 days of incubation period and inoculated with 100 µml/ 30 ml of media as initial inoculums size. Under optimal conditions, the glutaminase production improved to 2777 U/ml. The results of the present study indicatedt hat *Fusarium oxysporum*has immense potential as an industrial and economic organism for the production of L-glutaminase as extracellular enzyme employing submerged fermentation.

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