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OPTIMIZED PRODUCTION OF L-ARGINASE: A TUMOUR INHIBITOR ISOLATED FROM MARINE BACTERIA

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ABSTRACT

Our objective is to study l-arginase production pattern under submerged fermentation using novel marine isolate Idiomarina sediminum; H1695 (Gene Bank Accession Number JF346667) . Idiomarina sediminum; H1695 is a gram negative rod shaped marine bacteria isolated from chirala beach of Andhra Pradesh . It was screened for the enzyme production in mineral arginine medium. Further the activity was confirmed calorimetrically by estimating the levels of urea . The potential of an isolated novel strain for L-arginase production was analyzed under submerged fermentation with different process parameters and medium constituents. The maximum yield of enzyme production (215.36U/ml) was achieved in a seawater based medium at pH 9, 37°C, 10% inoculum concentration and 2% l-arginine concentration for 120 h. The medium when supplemented with carbon source, it improved the enzyme production from 121.49 to 158.37 U/ml with 1.5% maltose. Addition of 2% casein also improved the L-arginase production (168.23) .The overall production of the enzyme after optimization was increased to 215.36U/ml which is twice the original. This novel strain has immense potential as an industrial organism for the production of L-arginase as extracellular enzyme employing submerged fermentation. Further, it was observed that from the course of the present study, sea water could be used as an ideal fermentation medium for L-arginase production

KEYWORDS: L-arginase, Idiomarina sediminum; H1695, argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL).

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INTRODUCTION

L-Arginase (arginine amidinase, canavanase, L-arginase, arginine transamidinase EC 3.5.3.1) is a manganese-containing enzyme that catalyzes the deamidation of L-arginine to l-ornithine and urea [1]. Arginase is widely expressed in various worms, molluscs, fishes, bacteria, fungi, yeast, actinomycetes, algae and plants[2,3,4,5]. It is final enzyme in the urea cycle. It was found to counteract wide range of arginine auxotrophic cancers, such as melanoma, lung cancer, renal cell carcinomas and hepatocellular carcinomas [7,8,9,10] by nutrient depletion therapy. The enzyme has also been found to possess profound therapeutic benefits in treatment of various physiological disorders in the body. Measurement of circulating Arginase I i.e., serum arginase levels have been used experimentally as a rapid marker for liver injury [11]. Arginase has been found to be essential for the treatment of acute neurological disorders[12]. Ornithine, produced by arginase is necessary for the production of collagen, is helpful in therapy of rheumatoid arthritis [13]. Arginase upregulates the synthesis of polyamines and proline via arginine hydrolysis thus, being necessary to provide compounds for cell proliferation and growth [14]. Arginase competes with NOS for endogenous arginine pools, this way its levels acts as an indirect regulator of penile and vaginal flow thus playing an important role in male and female sexual arousal [15]. Upregulation of endogenous arginase I causes the activation of neural regeneration pathways, the reaction being mediated by polyamines and leading to novel roles of arginase in cell survival, regeneration and translation in the central nervous system [16]. Increase in psychological stress results in raising NO level which results in poor sperm quality. Thus, arginase levels are important to keep NO levels (via arginine degradation) in control for maintainence of semen quality[17]. Arginase has been shown to be effective for treatment of Hepatitis- B 139. Maarsingh et al., and Munder have reported arginase to be a key enzyme in pathophysiology of allergic asthma opening novel therapeutic roles for asthma control through action of arginase inhibitors [18,19] 187,188. Another important application of L-arginase is in biosensors for monitoring arginine levels in juice samples (10-2- 10 -5 M) and maximum concentration was found in orange juice [20] (10 -2 M). Several microbial strains having potential in the production of this enzyme , have been isolated and characterized at the growth and enzyme productivity level. The literature reports suggested that the enzyme produced by different microbial strains 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 usage sectors. Keeping in view, the great commercial application of L-arginase as a therapeutic agent; an attempt was made to optimize the composition of the L-arginase production media for the novel isolate , Idiomarina sediminum.

MATERIALS AND METHODS

Microorganisms

The halophilic bacterial species were isolated from chirala beach of Andhra Pradesh, India and was identified as Idiomarina sediminum; H1695 by 16S RNA sequencing method .It was shown to be a potential source of L-arginase and was employed in the present study. The slants were sub cultured at monthly intervals and stored at 4^0C in the refrigerator.

Cultivation medium and cultural conditions

The selected organism was cultured in production medium. The production medium consists of L-arginine - 20g**,** K2HPO4 -1, KH2PO4-0.1, MgSO4 -1, NaCl - 0.5, yeast extract -0.5, Aged sea water - 1000 ml. Ph - 8.

Optimization studies for biosynthesis of L-arginase

Required amount of the ingredients were weighed and dissolved in the adequate amount of water and volume was made up to 1000ml. The pH of the medium was adjusted to 6 using acid and alkali solution. Then the flasks were plugged and sterilized by autoclaving at 121^0 C for 20 minutes. 100ml of the production medium was transferred in

250ml Erlenmeyer flasks, inoculated and incubated for 24-48 hours at 35^0C.

Extraction of enzyme from production medium

The samples were withdrawn periodically at 24 hrs in aseptic condition. The extract was filtered through Whatman filter No.1. The clear extract was centrifuged at 2000-3000 rpm for 15 min, supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for assay of L-arginase.

Enzyme Assay Procedure

Arginase activity was measured in terms of the rate of hydrolysis of L- arginine to l-ornithine and urea by measuring the amount of urea released in the reaction. Urea was quantitated colorimetrically by the method of Archibald[21] . The reaction mixture consisted of 0.2 ml of glycine buffer (pH=9.5), 0.5 ml of appropriately diluted enzyme and 0.1 ml of manganese chloride. After incubation at 37ºC for 10 min, the enzyme assay was initiated by adding 0.1 ml of arginine and the mixture was further incubated at 37ºC. After 30 min, the assay was stopped by adding 1 ml of perchloric acid solution. The total volume of the samples was adjusted up to 2.0 ml. with distilled water. To this 1.0 ml of the phosphoric acid/sulphuric acid mixture (3:1) was added & the contents were mixed well. Then,0.1mlof 4%α-isonitrosopropiophenone was added and the tubes were capped . The tubes were then placed in a boiling water bath. After 1 hour, the solution was removed & cooled at room temperature. The absorbance was taken at 540nm. The urea produced was estimated from urea curve prepared by varying the concentration of urea between 0.1 to 1 µmoles and a graph of optical density against urea concentration was plotted for the extrapolation of L-arginase activity.One unit of enzyme activity is defined as the amount of enzyme that catalyses the release of 1µmol of urea at 37^0C.

Determination of enzyme protein

Enzyme protein was measured according to the method of Lowry et al., (1951) using Folin Ciocaltaeu's reagent.

Optimization Studies for biosynthesis of L-arginase

To optimize various parameters for L-arginase production at shake- flask level, different components of the media and other enzyme production conditions were replaced and tested. Each experiment was performed in triplicate and the enzyme produced was assayed by standard assay procedure of Archibald [21]. Following optimization, a well defined production medium was developed for efficient enzyme production. Various physical and chemical parameters were optimized for the production of L-arginase at shake-flask level. The following parameters were studied:

Effect of incubation time

The effect of incubation time (0-144 hrs.) on L-arginase production was studied.

Optimization of inoculum size for L-arginase production

To study the effect of inoculum size on L-arginase production, erlenmeyer flasks containing 100ml mineral arginine media (MA) were incubated with inoculum sizes ranging from 2.5%, 5%, 7.5%, 10%, 12.5% and 15% inoculum. Following growth of micro-organism, enzyme was extracted and assayed for activity.

Optimization of inoculum age for L-arginase production

Erlenmeyer flasks containing 100ml MA media were incubated with inoculums having inoclum ages of 9hrs, 12hrs, 15hrs, 18hrs, 21hrs, 24hrs, 27hrs and 30 hrs. Optimum inoculum age for further culturing was selected by means of assay of enzyme produced in various flasks inoculated with inoculum of different ages.

Selection of Carbon sources for L-arginase production

Erlenmeyer flasks (250 ml) containing 1%(w/v) of various carbon sources such as glucose, cellobiose, maltose, sucrose, xylose and arabinose, were inoculated for arginase production to optimize the best carbon source for arginase production. The flasks were autoclaved, inoculated and incubated. The enzyme was extracted and assay.

Effect of various concentrations of best carbon source on the production of L-arginase

To optimize, the effect of various concentrations of best carbon sources on the production of L-arginase was studied.

Selection of Nitrogen sources for L-arginase production

After optimization of the carbon source and its concentration, various alternative nitrogen sources i.e., peptone, tryptone, yeast extract, ammonium chloride and ammonium nitrate (1% w/v) were substituted singly in the medium. The enzyme was extracted and assayed.

Optimization of casein concentration for L-arginase production From the above experiment, it was observed that casein was the best nitrogen source for optimum enzyme production. Hence different concentrations of casein (0.5%, 1%, 2% and 3% w/v) were supplemented with the mineral arginine media for optimization of concentration for the maximum production of L-arginase. Inoculation was carried out at 10% (v/v) and incubation was done under optimized conditions. The enzyme production was measured by the standard assay procedure.

Selection of inducer for the L-arginase production

Using maltose and casein as carbon and nitrogen sources, respectively, various inducers such as L-arginine, L-asparagine, L-glutamine, L- ornithine, and D-arginine were tested (1% w/v) for optimum enzyme.

Optimization of L-arginine concentration for L-arginase production

Different concentrations of L-arginine (0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 1.0% w/v) were supplemented to the mineral arginine media for optimization of concentration for the maximum production of L-arginase.

Effect of different NaCl concentrations

NaCl is an essential constituent of the arginine medium. Its concentrations were varied from 0.1-19% to know a possible effect on enzyme production.

Effect on pH

L-arginase has been reported to be an alkaline enzyme with a few references available on its activity in acidic environments as well. Thus, its production was studied under different pH conditions ranging from acidic, neutral and alkaline conditions from pH 6 to pH 11 (pH values –6.0, 6.5, 7.0, 7.2, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 &11.0).

Effect of Temperature

Temperature conditions were varied to optimize the best temperature for efficient enzyme production. Temperature ranging from 25^{o} C to 45 ^{o} C was used to measure its effect on L-arginase titer obtained.

Effect of Shaking condition

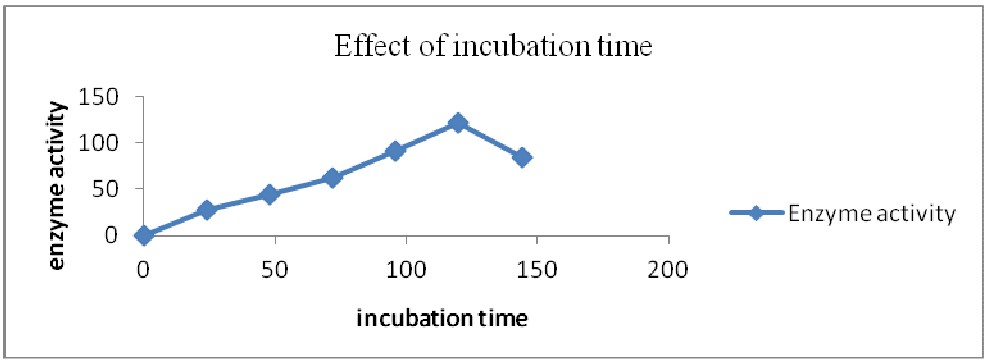
The culture was subjected to different shaking conditions ranging from 25-250 rpm and its effect on L- arginase production was noted down.

RESULTS

Effect of incubation time

Different incubation time (0-144hrs) were adopted to determine the optimum one for L-arginase production. Maximum production of enzyme was seen after 120hrs of incubation.

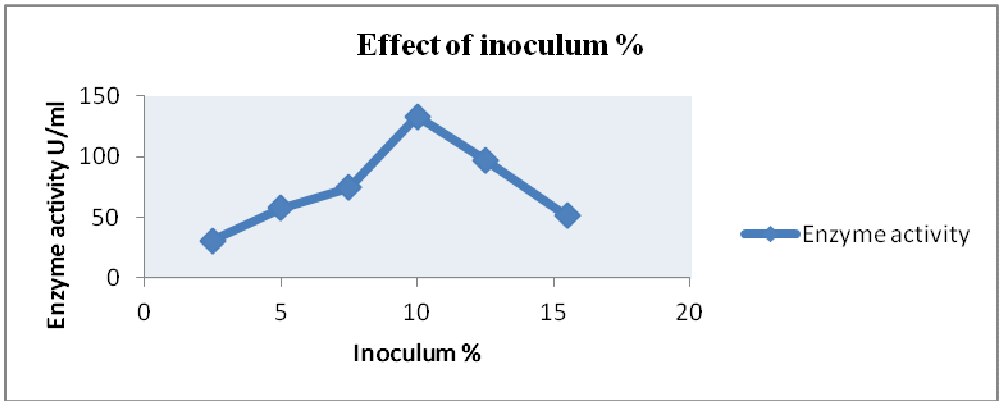
Figure 1
Effect of incubation time on L-arginase production



Optimization of inoculums size for the L-arginase production

Starter culture was inoculated in mineral arginine media at various concentrations of inoculum [2.5, 5, 7.5, 10, 12.5 and 15 % (v/v)] . There was increase in L-arginase production as inoculum size was increased from 2.5-10% but beyond that L-arginase activity decreased further. The results are shown in & Figure 2.

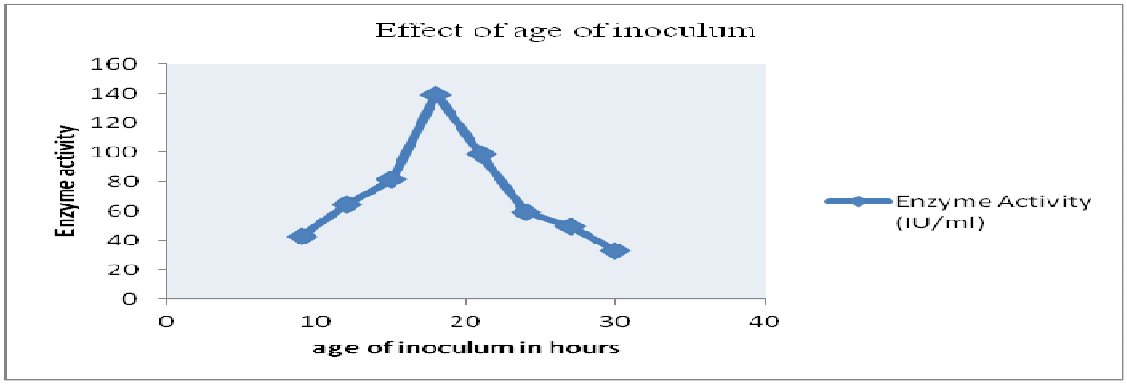
Figure 2
Effect of inoculum % on L-arginase production



Optimization of inoculum age for the L-arginase production

Inoculum (10 %) of different age (9, 12, 15, 18, 21, 24, 27 and 30 hrs) was introduced in mineral arginine media. The best inoculum age to give the most favorable results in terms of both yield and productivity of the enzyme was found to be 15hours. The results are shown in Figure 3.

Figure 3
Effect of inoculum age on L-arginase production

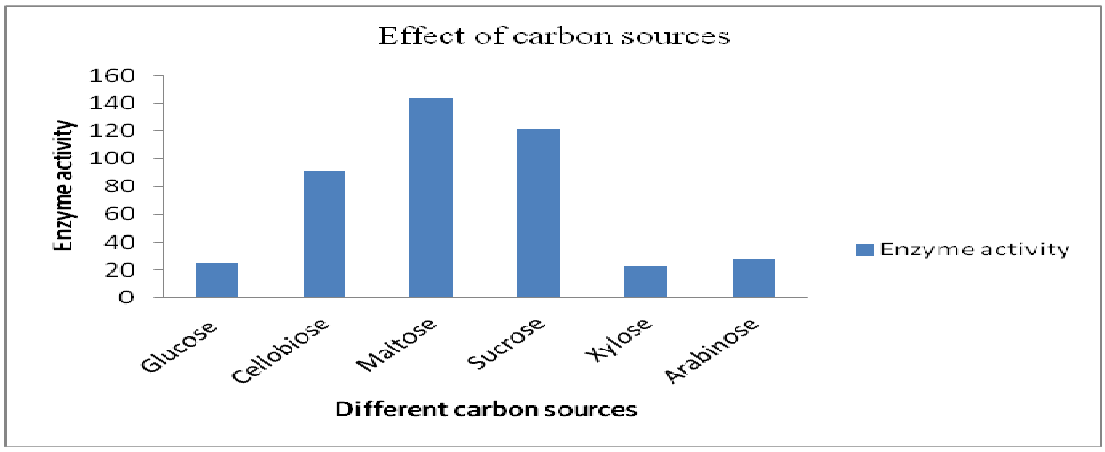


Selection of carbon sources for the L-arginase production

An important aspect for the growth and enhanced production of L-arginase is the nature and amount of carbon sources in the culture medium.Out of all the carbon sources added, maltose was the most effective source of carbon. A maximum enzyme activity of 143.56 IU/ml was achieved (Figure 4). Idiobacterium sediminum is a heterotrophic bacteria and depend upon organic sources for energy . It is having ability to use limited carbon sources.

Figure 4

Effect of carbon sources for the L-arginase production

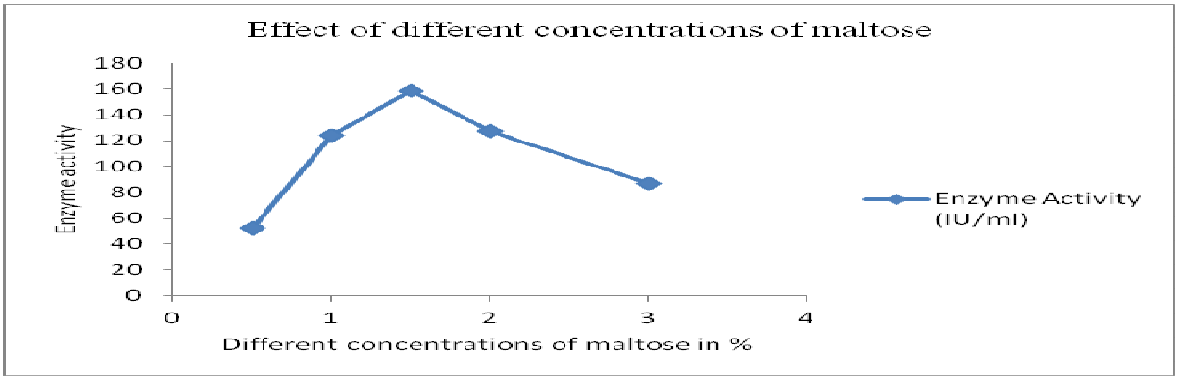


Optimization of maltose concentration for the L-arginase production

In the previous experiment it was observed that maltose is the best carbon source for the L-arginase production. Various concentrations of maltose (0.5, 1, 1.5, 2 and 3 %, w/v) were used in the fermentation medium and the maximum enzyme activity of 158.37 IU/ml (Figure 5) was achieved with a maltose concentration of 1.5% (w/v).

Figure 5

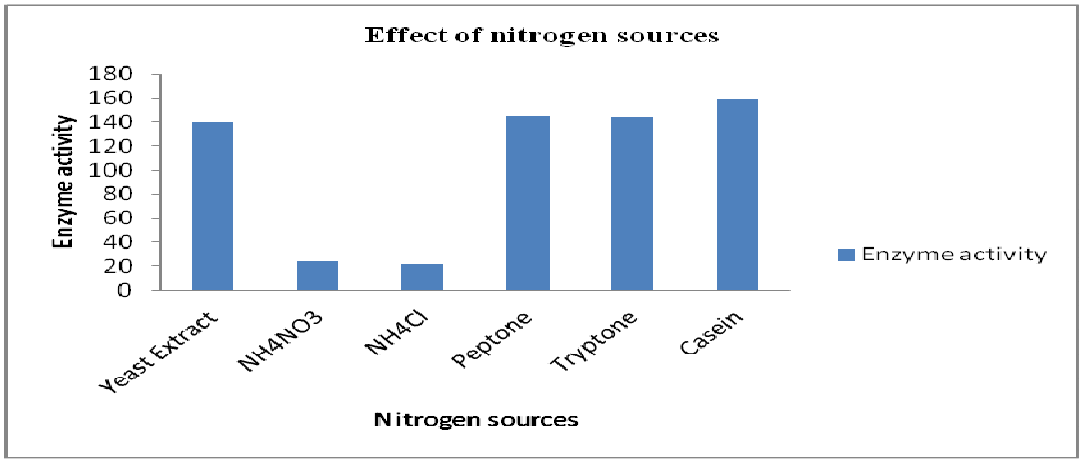
Effect of maltose concentration on L-arginase production



Selection of nitrogen sources for the L-arginase production

After optimization of the carbon source and its concentration, various alternative nitrogen sources [peptone, tryptone, yeast extract, ammonium chloride, ammonium nitrate (1% w/v),casein] were substituted singly in the medium to know the best nitrogen source for optimum enzyme activity, in the presence of maltose (1.5%), as a carbon source.Casein was found to be the best nitrogen source for L-arginase production.

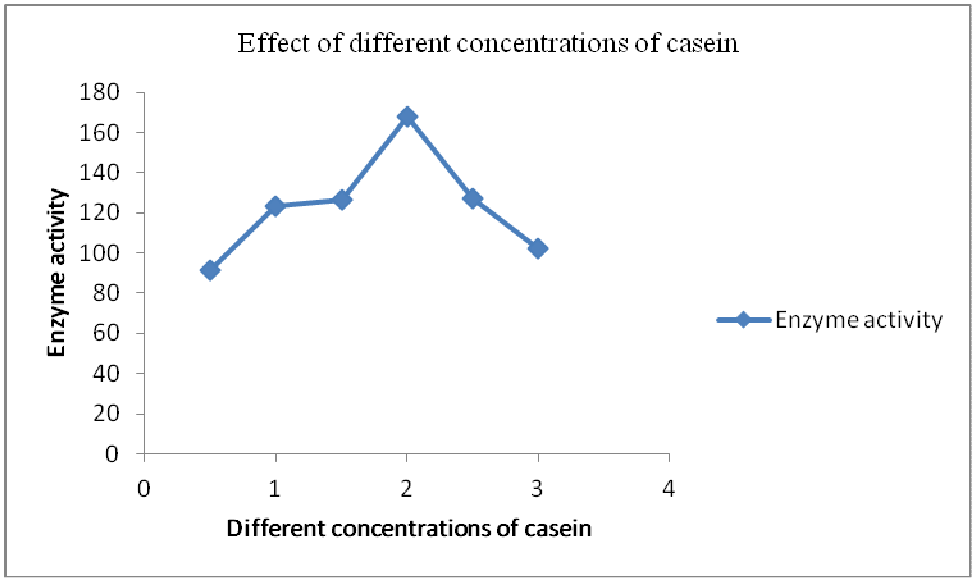
Figure 6
Effect of nitrogen sources on L-arginase production.



Optimization of casein concentration for the L-arginase production

Different concentrations of casein (0.5, 1, 2, 3 and 4% w/v) were supplemented to the mineral arginine media for optimization of concentration for the maximum production of L-arginase. The results are shown below in figure 7.

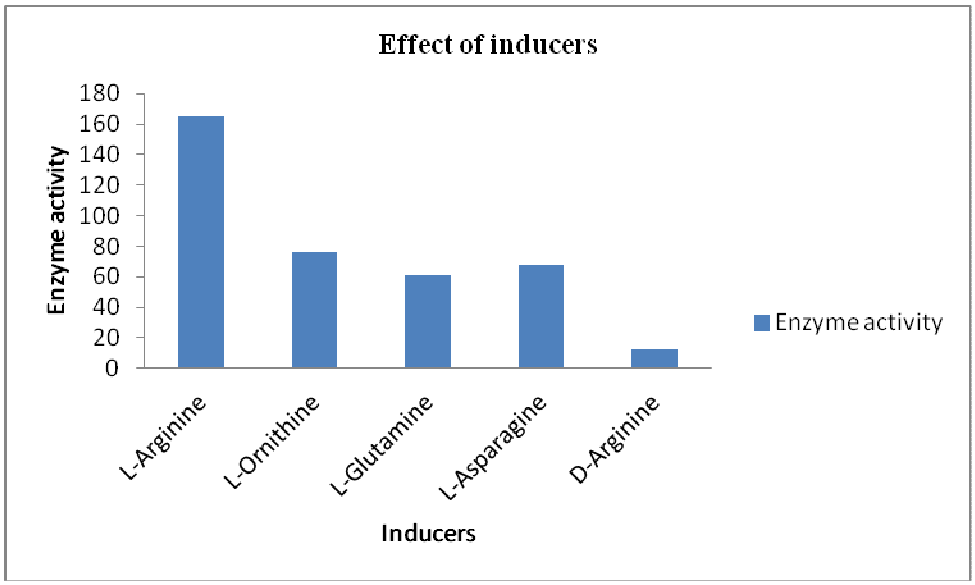
Figure 7
Effect of casein concentration on L-arginase production



Selection of inducer for the L-arginase production

After the optimization of carbon and nitrogen source, different inducers such as L-arginine, L-asparagine, L-glutamine, L-ornithine, D-arginine were used for the production of L-arginase in the presence of maltose and casein; as a carbon and nitrogen source respectively. Out of the various inducers tested, L-arginine was found to be the most effective for efficient enzyme induction. The enzyme produced has been found to be quite specific for L- arginine as its substrate, as can be seen from Figure 8 where very little activity has been found incase of D-arginine when used as a substrate.

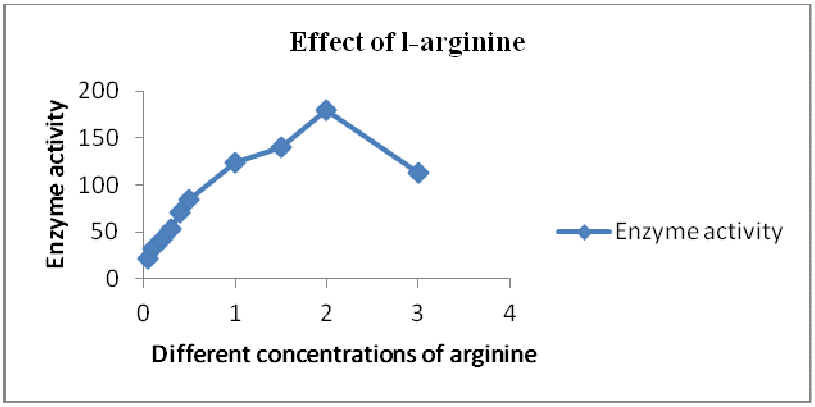
Figure 8
Effect of inducer on L-arginase production



Optimization of L-arginine concentration for L-arginase production

For our study, L-arginine concentrations ranging from 0.1% to 3 % were tested to know the optimum percentage for enzyme induction and the best results for enzyme production were obtained at 2% arginine as an inducer as shown below in figure 9.

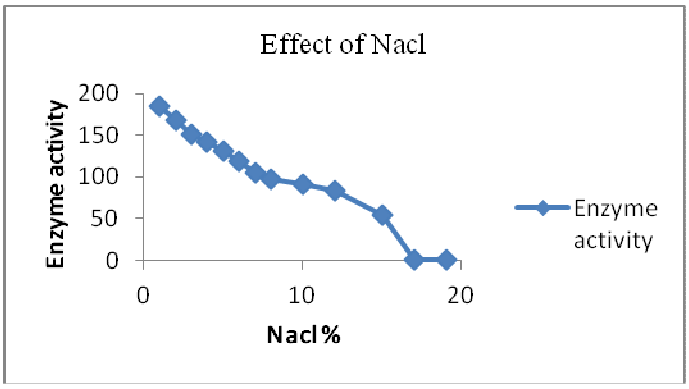
Figure 9
Effect of L-arginine concentration for L-arginase production



Optimization of NaCl concentration for the L-arginase production

NaCl is an important constituent of the minimal arginine medium. Its concentrations were varied from 0.1-15% to know a possible effect on enzyme production. 2.5% NaCl concentration gave the maximum enzyme titer as shown in Figure 10.

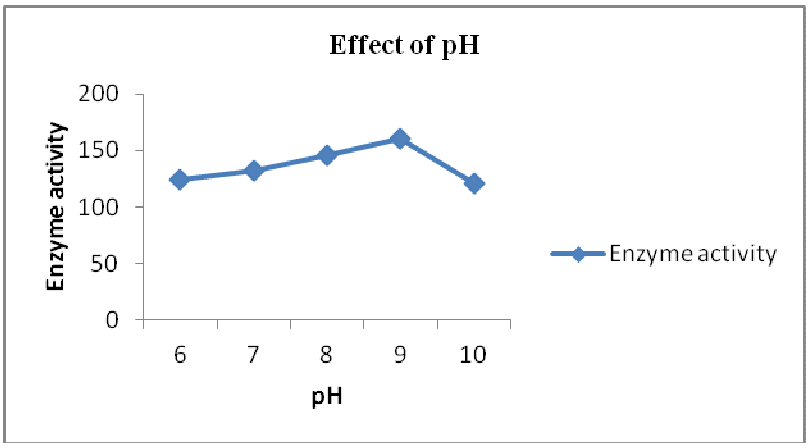
Figure 10
Effect of NaCl concentration on L-arginase production



Effect of pH on the production of L-arginase

Arginase showed maximum activity at pH 9 . Production of enzyme was seen between 6-10 . As can be seen Figure 11, the enzyme started losing activity when subjected to pH greater than 11.

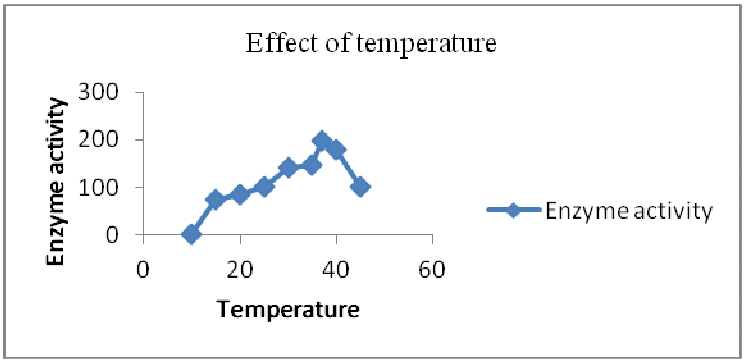
Figure 11
Effect of pH on the production of L-arginase



Optimization of temperature for the L-arginase production

L-arginase showed maximum activity at a temperature of 37° C. However it was quite stable and active at temperatures of 30°C & 40°C. As can be seen in Figure 12, the enzyme started losing activity when subjected to temperatures greater than 40°C.

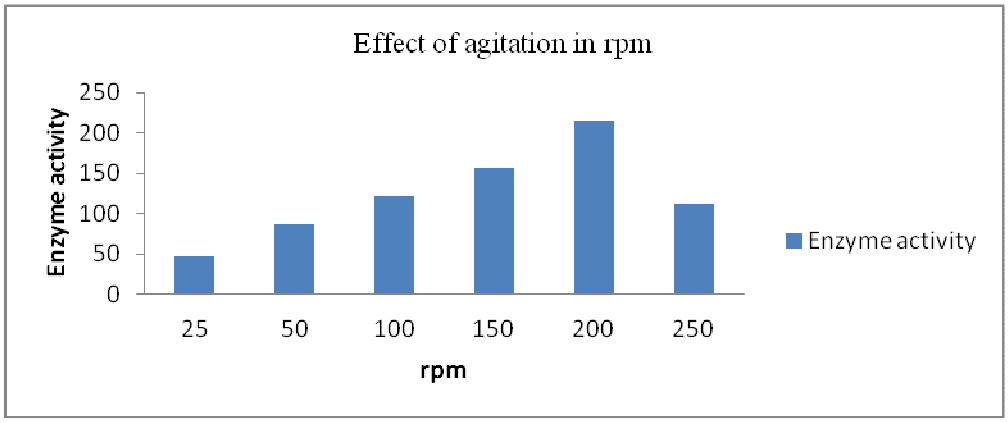
Figure 12
Effect of temperature on the L-arginase production



Optimization of agitation for the L-arginase production

Each 250 ml fermentation flask containing 100 ml medium was subjected to different shaking conditions like 25,50, 100, 150, 200 and 250 rpm to know the effect of shaking on L-arginase production. The results can be seen in Figure 13

Figure 13
Effect of agitation for the L-arginase production



DISCUSSION

Incubation time, Inoculum size & inoculum age

The organism produced L- arginase in the L-arginine containing production medium .The enzyme production showed growth relatedness as the incubation period progressed and the maximum enzyme production was observed after 120 hrs (5days) of incubation (Figure 1). After 5 days the enzyme production decreased as the growth of microorganisms might have reached a stage where the organisms could no longer remained in the balanced state of the growth with the available nutrients in the medium or enzymes might be inactivated.10 % of inoculum size and 15 hrs of inoculum age was found to be optimal for L-arginase production.

Carbon source & nitrogen sources

Idiomarina sediminum is a heterotrophic bacteria , derives energy from organic sources. It was seen that it is having ability to use limited carbon sources. Of various carbon sources , maltose has been found to be the best carbon source for L-arginase production followed by sucrose . Various concentrations were tested to know their effect on enzyme production. Out of the various concentrations of maltose, appreciable enzyme activity was found at 1.5%. The results are shown in Figure 4 & 5. The arginine catabolic pathway of organism which proceeds via citrulline and is induced by arginine has been reported to be non-induced in the presence of glucose. In our study glucose has been found to repress L-arginase activity . Various nitrogen sources - yeast extract, peptone , casein, tryptone , NH4NO3 and NH4Cl were chosen and were tried for getting favorable nitrogen source to produce L-arginase. Casein (2%) supported L-arginase production more than other nitrogen sources which reflects the efficiency of this nitrogen source (in the combination with arginine) to provide growth requirements and production of L-arginase, due to its composition as a protein, supplies essential amino acids for construction of many important compounds in the cells included enzymes. Next best nitrogen source was peptone & tryptone. NH4Cl and NH4NO3 were found to be simple nutrients, hence do not enhance the enzyme activity.Whereas tryptone, casein etc are complex nutrients provide a mixture of nutrients for the optimal production of enzyme.

Incubation temperature , inducers, pH

The results indicated that L-arginase is produced in a wide range of temperatures i.e. 13 to 42^0C, maximum production of L-arginase was at 37ºC; other temperatures diminished the enzyme productivity. This may

attributed to that this isolate favores this temperature for growth and metabolism. After the optimization of carbon and nitrogen source, different inducers such as L-arginine, L-asparagine, L-glutamine, L-glutamate, L-ornithine, D-arginine were used for the production of L-arginase in the presence of maltose and casein; as a carbon and nitrogen source respectively. Out of the various inducers tested, L-arginine was found to be the most effective for efficient enzyme induction. The enzyme produced has been found to be quite specific for L- arginine as its substrate, as can be seen from Figure 8 where very little activity has been found incase of D-arginine when used as a substrate. Arginine acts as an effective inducer of the enzyme L-arginase in a concentration of 2%. To examine the effect of pH on the L-arginase production, pH of the growth medium was varied . Arginases from various sources ranging from microbial to mammalian sources have been reported to be mostly alkaline enzymes having pH optima ranging from pH 6-10. Optimal growth of the organism occured between 8-9.Agitation speed of about 200rpm shown to be optimum for the enzyme production, since agitation provides equal distribution of nutrients and gases (oxygen) to the microorganisms.

CONCLUSION

The potential of an isolated novel strain Idiomarina sediminum; H1695 (Gene Bank Accession Number JF346667) for L-arginase production was analyzed under submerged fermentation with different process parameters and medium constituents. Maximum production was noticed at pH 9, 37°C, 200 rpm, with 10% inoculums, 2% arginine concentration, 1.5% maltose, 2% casein and 120 h of incubation period. Under optimal conditions, the L-arginase production improved to 215.36U/ml which is almost twice the original yield. The results of the present study indicate this novel strain has immense potential as an industrial organism for the production of L-arginase as extracellular enzyme employing submerged fermentation. Further, it was observed that from the course of the present study, sea water could be used as an ideal fermentation medium for L-arginase production.

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