Research Article

Screening, Production and Characterization of extracellular glutaminase free L-Asparaginase producing endo-symbiontic bacteria from the gut of *Mugil cephalus* (Mullet fish)

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Abstract

Objective: Gut microbes producing L-asparaginase isolated from marine fishes has proven to be one of the best anticancer agent in case of Acute Lymphoblastic Leukemia (ALL). Hence this study focuses on extracting the L-asparaginase producing microbe from the gut of *Mugil cephalus*. **Materials and methods:** The microbes were isolated, screened, identified for maximum enzyme production and the medium was subjected to various optimization processes. **Results:** L-asparaginase activity was detected on the basis of formation of red colour around the colony. The high yielding strain (LAS-8) was grown in Modified Czapek Dox media with different carbon sources and glycerol showed the maximum enzyme production (39.53IU/mg). Optimization with different pH and temperature resulted in maximum production at pH (6.5-7) and temperature 30°C-40°C. The antibiotic sensitivity test was performed in order to identify whether the microbe is susceptible to antibiotics. **Conclusion:** Findings of this study suggest that the L-asparaginase isolated from the fish gut can be an effective treatment for Acute Lymphoblastic Leukemia (ALL).

Keywords: Enzyme, L-Asparaginase, *Mugil cephalus*, Acute Lymphoblastic Leukemia (ALL)

Introduction

Intense research has been carried out in the fish intestine since it is a multifunctional organ and is central to nutrient uptake, pathogen recognition and regulating the intestinal microbiome (Martin et al., 2016). Enzyme production by microorganisms has been used in various industries in the world. Microbial L-asparaginase has been widely used as a therapeutic agent in the treatment of certain human cancers, mainly in acute lymphoblastic leukemia (Bhargavi and Jayamadhuri, 2016). The reason for the purpose is it is bio-degradable, non-toxic and can be easily administered at the local site (Kamble et al., 2012). L-asparaginase is of special significance because of the fact that tumor cells are deficient in L-asparaginase synthetase activity, which restricts their ability to synthesize non-essential amino acid L-asparagine, required for the growth and survival of cancer cells. Therefore, tumour cells are dependent on exogenous supply of L-asparagine from body fluids (Ahmad et al., 2012). The enzyme catalyzes the hydrolytic reaction of L-asparagine into L-aspartic acid and ammonia (Saxena et al., 2015). Bacterial L-asparaginase has been the subject of considerable medical interest and is being employed in the therapy of acute lymphoblastic leukemia. The present study focuses on extracting the L-asparaginase producing microbe from the gut of *Mugil cephalus*.

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Materials and methods

Collection of samples

Mugil cephalus was collected from the Velrampet lake (Latitude: N 11° 54.9217' Longitude: E 79° 48.2542'), South east coast of India, Pondicherry and transformed to sterile polythene bags using forceps. The fresh sample was immediately transferred to the laboratory and subjected to various analyses.

Isolation of the gut and their homogenization

Fish was anaesthetized in an ice bath for 10–15 min and was

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surface sterilized by immersion for 40 seconds in 70% ethanol. Aseptically the gut was dissected from the animal's musculature. It was weighed and placed into a 10 ml sterile double strength phosphate-buffered saline solution (PBS) (disodium phosphate, 2-3% (w/v); sodium phosphate, 0-6% (w/v) and sodium chloride, 1-2% (w/v)). Gut was homogenized in tissue homogenizer.

Isolation of marine bacteria

Employing standard microbiological methods (Zobell, 1947), gut dissected from fish was grinded using sterile mortar and pestle, serially diluted and plated on Zobell marine agar supplemented with 3% NaCl contained in sterile petri plates and incubated at 30ºC for 24 h. The bacterial isolates were selected at random on the basis of their colony morphology. Based on their capacity to produce potential L-asparaginase, these bacterial isolates were chosen for preliminary screening studies.

Screening of isolated marine bacteria for L-asparaginase production

Semi-quantitative rapid plate assay was used to screen the bacterial isolates for the production of L-asparaginase (Gulatti et al., 1997). On the modified M9 medium the cultures were streaked supplemented with L-asparagine (2% w/v) and phenol red (0.09% w/v) as pH indicator. On incubation, phenol red appears yellow in colour at acidic pH and turns to pink in alkaline pH. After 48 h of incubation the colonies producing the pink zone were screened as positive strains producing L-asparaginase (Noura El-Ahmady et al., 2016).

Determination of L-asparaginase activity

The hydrolysis of L-asparagine is catalyzed by L-asparaginase to form L-aspartic acid and ammonia. The quantitative estimation of L- asparaginase production was measured by Nesslerization reaction, based on the estimation of ammonia liberated during the process (Bauer et al., 1966). All the active strains were grown in M9 broth supplemented with L-asparagine (2% w/v) and Sodium chloride (3%) for 48 h at 30ºC. The cultures were centrifuged at 10,000 rpm for 15 min and the supernatant was collected and were used as crude extract for the determination of the L-asparaginase activity. The reaction mixture containing 0.5 ml of 0.04 M L-asparagine, 0.5 ml of 50 mM, Tris buffer, 0.5 ml of the enzyme preparation and the final volume was made upto 2.0 ml, incubated at 37º C for 30 min. Then the reaction was stopped by the addition 0.5 ml of 1.5M Trichloro acetic acid (TCA). Blank was prepared by adding enzyme preparations after the TCA is added. The precipitated proteins were removed by centrifugation. To 3.7 ml of distilled water 0.1 ml of the above mixture and 0.2 ml of Nessler's reagent were added and incubated at 20ºC for 15 min. The absorbance was measured at 450 nm using spectrophotometer and the amount of ammonia liberated was determined. One international unit of L-asparaginase is defined as the amount of enzyme that liberates 1 μM of ammonia per minute under the assay conditions. Further the influence of different carbon source and concentration of selected carbon source on L-asparaginase production were also evaluated for the isolate LAS-8.

Submerged fermentation

Submerged fermentation carried out for the active bacterial strains using 250ml capacity Erlenmeyer flasks, containing 100ml of glycerol asparagine medium. Each flask was inoculated with 1ml culture suspension (three days old). Inoculated flasks were incubated at 30°C for five days on a rotary shaking incubator at 250 rpm. Samples were taken periodically every day for determination of L-Asparaginase activity. After incubation the fermented media was centrifuged at 10,000 rpm or 20 min for crude enzyme preparation.

Qualitative Screening of the microbes

153 organisms were isolated from the fish gut. Of that 16 isolates have shown positive results for asparaginase production. 8 isolates have shown optimum enzyme production and isolate 8(LAS-8) has proven to produce the maximum enzyme production.

Antibiotic Sensitivity Test

Antibiotic sensitivity test was performed by standard procedures. The isolate LAS-8 was spread by lawn culture and the antibiotic discs were placed. Zone formation around the disc infers that the microbe is sensitive to antibiotics hence it is not pathogenic (Bauer et al., 1966).

Molecular characterization

Genomic DNA was extracted and PCR was performed with Universal bacterial primer.

Sequencing of 16s rRNA of the asparaginase producing strain was done with BigDye Version 3.1 kit (Applied Bio-systems) on an ABI-PRISM 3730 DNA Sequencer (Applied Bio-systems).

Statistical analysis

The values are means of triplicate readings ±SD. The statistical difference between the groups was determined by ANOVA. The level of $p<0.05$ was considered to be statistically significant.

Results

Screening and isolation of bacteria

The bacterial isolates from the fish gut were subjected to quantitative analysis of enzyme production in order to

identify the high yielding strain. 8 strains were found to produce L-asparaginase, out of them strain 8 (LAS-8) showed maximum enzyme production of 69.88 IU/mg which is evident from Figure1.

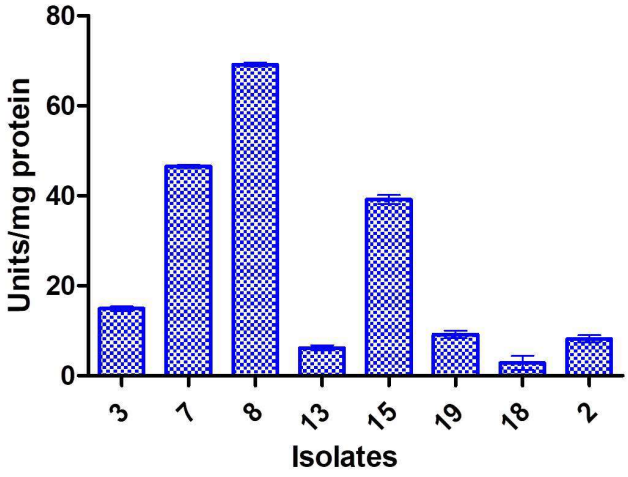

Figure 1. Quantitative Screening of Asparaginase producing bacterial isolate

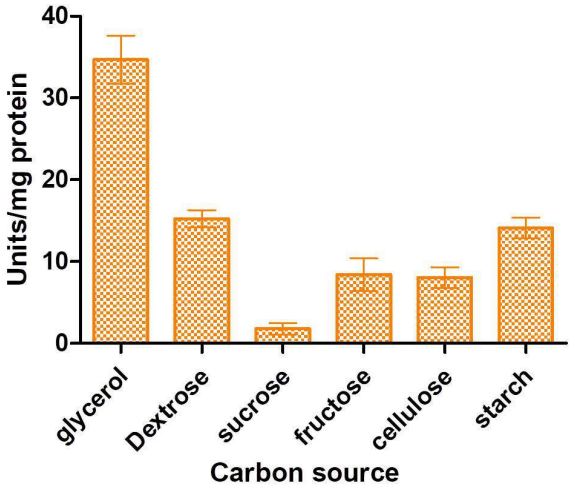

Figure 2. Screening of different carbon sources for asparaginase production

Optimization of media components for L-asparaginase production

Effect of different carbon sources on enzyme production

The carbon source plays an important role in the media composition in the enzyme production. Different carbon sources such as glycerol, dextrose, sucrose, fructose, cellulose and starch were subjected to media optimization for L-asparaginase production. Among them glycerol has shown the maximum enzyme production (39.53IU/mg). Dextrose and starch exhibit similar quantity of enzyme production 17.06IU/mg and 16.67IU/mg respectively. Similar standard was observed in fructose and cellulose (11.87IU/mg and 10.26IU/mg) and the least production was observed in the sucrose (2.99IU/mg).

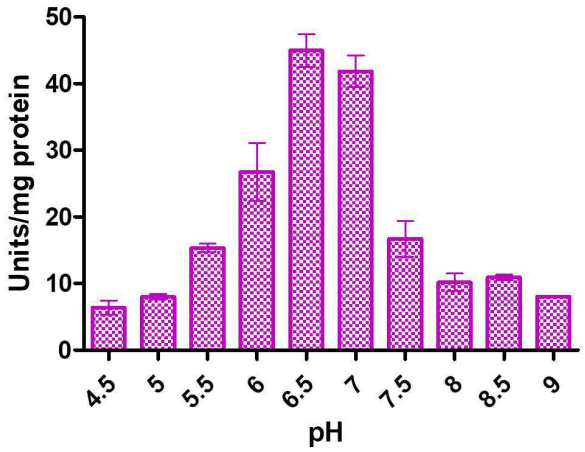

Figure 3. Optimization of pH for enzyme production

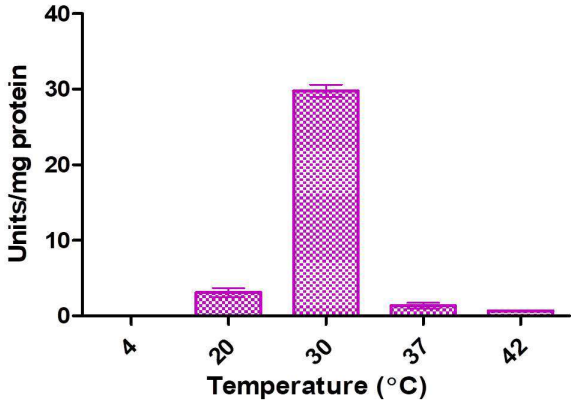

Figure 4. Optimization of temperature for enzyme production

Optimization of pH for asparaginase production

The asparaginase production showed gradual increase from lower pH of 4.5 to neutral pH of 6.5-7 (3.63-44.98IU/mg) and the production started decreasing when the pH is increased to 9. From figure it is evident that the maximum production (35.39IU/mg) was obtained in the pH 6.5-7 which showed that the gut microbe was not halophilic in nature. The least value (4.53IU/mg) was recorded at acidic pH of 4.5.

Optimization of temperature for asparaginase production

Depending upon the temperature the amount of enzyme production varies. The figure showed at freezing temperature (4°C) there is no evident enzyme production whereas the maximum production was observed at 30°C (29.89IU/mg) and higher temperature of 40°C showed least enzyme production (2IU/mg). Hence at extreme conditions the bacterial isolate fail to show enzyme activity.

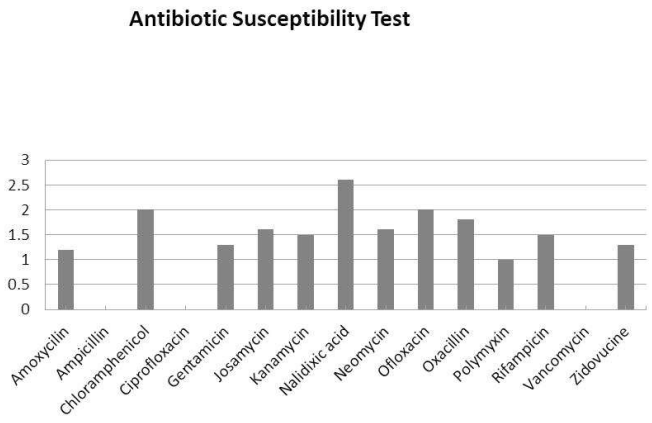

Figure 5. Effect of different antibiotics on LAS-8

Antibiotic sensitivity test

Antibiotic susceptibility test was performed with 15 antibiotics in order to confirm whether the organisms are susceptible to antibiotics. LAS-8 was susceptible to 13 antibiotics tested and it is resistant against 3 antibiotics.

Molecular characterization of the potent asparaginase producing strain

16srRNA sequencing of the potent strain isolated from the gut of *Mugil cephalus* showed that the sequence of SUB4055578 had homologous similarity with *Enterobacter cloacae S1*.

Mh368114 is the accession number allotted by Genbank for the submitted nucleotide sequence.

Discussion

Mugil cephalus is cosmopolitan in the coastal waters of most sub-tropical and tropical zones. The flattened grey mullet is frequently found coastally in estuaries and freshwater environments. Flattened grey mullet is a diurnal feeder, dead plant matter, consuming mainly zooplankton and detritus (FAO's, 2018). L-asparaginase has proved to be effective anti-Lymphoblastic Leukemia in children (Verma et al., 2007). Fish gut microflora plays an important role in the growth, digestive process and disease of the host (Olsson et al., 1992).

In the present study, L-Asparaginase producing gut microbes were isolated. The qualitative analysis of the 8 isolates showed pink zones in Modified Czapek Dox medium when adding phenol red as indicator. This result was in accordance with the work done by Sarquis et al., (2004) and Dhevagi and Poorani (2006). Based on the quantitative assay the strain that produces maximum enzyme was isolated. Further optimization was carried with LAS-8.

L-asparaginase production depends upon various factors such as temperature, pH, agitation, age of inoculum, incubation time etc., since the significant biosynthesis of L-asparaginase relies upon environmental factors. The optimum pH for L-asparaginase production by LAS-8 was 6.5 whereas maximum production from Bacillus sp. was reported at pH 8.0 (Mohapatra et al., 1995). Similarly optimum temperature for the L-asparaginase production was found to be 30° C this is in accordance with the work of Narayana et al., (2008). Among the different carbon sources used in the optimization process glycerol showed the maximum production by LAS-8. In contrast Sushmitha and Mandaal (2013) have reported sucrose as the best carbon source for Bacillus sp. and Amena et al (2010) reported maltose as the best carbon source for *Streptomyces gulbargensis*. The sensitivity of the strain LAS-8 to antibiotics was tested against 15 antibiotics. The growth of LAS-8 was highly controlled by Nalidixic acid followed by Chloramphenecol and Ofloxacin. It is not susceptible to Ampicillin, Ciprofloxacin and Vancomycin.

Molecular characterization and 16srRNA sequencing concluded that the sequence SUB4055578 submitted to GenBank had homology with *Enterobacter cloacae* with accession number MH368114.

Acknowledgments

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Conflict of interest

All authors have declared no conflict of interest.

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