INTRODUCTION

Current cancer treatments causing side effects on the human in some instances. Extracted natural products from medicinal plants considered as a significance cancer treatment. The marine organisms able to produce novel chemicals against extreme variations which are unique in diversity, structural, and functional features (Kathiresan et al., 2008). L-asparaginase was known as L-asparagine amidohydrolase (EC. 3.5.1.1). L-asparaginase is useful anti-leukemia enzyme because of its ability in hydrolyzing L-asparagine into aspartic acid and ammonia (Dias and Sato, 2016). In the proliferation of leukemic cell L-asparaginase is an important amino acid. Researchers mentioned that it can be isolated L-asparaginase producer from a wide diversity of microbe especially bacteria which are most studied because of the high stability of isolation as claimed (Sanjeeviroyar et al., 2010). Halomonas daceae, Halomonas is one of the genera most frequently isolated from hypersaline waters and soils by conventional-culture techniques (Ventosa et al., 2008; Kaye and Baross, 2000). The ecological role that Halomonas species play in these habitats and their relationships with other halophilicand non-halophilic microorganisms are still unknown. Most species of the genus Halomonas have been recognized as having potential applications in biotechnology due to their capacity to produce compatible solutes, enzymes or exoply saccharides and for their role in the degradation of pollutants, such as several aromatic compounds (Ventosa, 2006; Rafael et al., 2011; Oren, 2010).

MATERIALS AND METHHODS

Samples: Save marine samples were collected from the west coast of Saudi Arabia at red sea. At shore (Nerita sp.) and (Actotaeodea glabrate), from depth of 50 cm one species of brown algae (Dictyota sp.), one species of green algae (Uvalactuca) and at the depth of 20 m, one species of solitary discoral (Clenactiscrassu),and two species of soft coral (Sarcoponts p., Xenia umbellate).

Isolation of L-asparaginase producing bacteria: Suspensions were prepared by mixing 10g of sediment samples into conical flasks containing 100mL of sterile phosphate buffer, rotated at 50 rpm/min for 30min, the suspending matter and the clear supernatant was decanted and serially diluted. Further, 100UL of the different dilutions spread on Glucose Asparagine solid medium (glucose 20g, asparagene 5g, KCl 0.5g, K2HPO4 1.0g, MgSO4, 7H2O 0.5g and pH7.0±0.2 in 1000mL sea water). The medium was supplemented with phenol red (2.5%): 0.04-0.36 mL indicator. The plates were then incubated at 35±2°C for 24-72h, to obtain colonies with pink zones around them (Gulati et al., 1997).The more potent strains were selected for fermentation process.
Preparation of inoculums: The bacterial strain was inoculated in nutrient agar plates and incubated at 35±2°C for 24-48h. One or two colonies of bacterial growth were inoculated into 50 ml of nutrient broth in 250 ml flask and kept in a rotary shaker (150 rpm) at 35±2°C for 24h.

Estimation of L-asparaginase: Assayed through Nesslerization method described by (Imada et al., 1973). The measurements was conducted by spectrophotometer at 450 nm. The reaction mixture was assayed in triplicates (Basha et al., 2009).

Molecular characterization of bacterial isolate: Genomic DNA isolation using Gene JET Genomic DNA Purification Kit.

- PCR mixture: 2µL DNA, 1µL of each primer, 12.5µL Master mix and 9.5µL sterile dH2O.
- PCR amplification conditions: Initial: 5 min at 94 °C, Denaturation: 1 min at 94 °C, Annealing: 1 min at 55 °C and Extension: 2 min at 72 °C, Number of cycles: 35. Final extension: 10 min at 72 °C.

Optimization of L-asparaginase production by Halomonas alkaliantarctica

Effect of shaking incubation on L-asparaginase production: Two sets of cultures flasks; one was incubated in static incubation another on a rotary shaker 150rpm at 35±2°C. After 48h of incubation cultures filtrates were collected and centrifuged at 4°C for 30 min at 10000 rpm. The supernatant used as a source of the crude enzyme.

Carbon Sources: To investigate the effect of carbon sources on L-asparaginase (ASNase) production by H. alkaliantarctica, the carbon source(glucose) had been replaced by different carbon sources in AG broth medium such as lactose, sucrose, and glucose, each added at a concentration of 1% (w/v) (Amenaet al. 2010; Narayana et al., 2008), keeping other components constant.

Amino acids and other Nitrogen Sources: Deferrnt aminoacids, asparagine, arginine, glutamine, glycine and methionine, and nitrogen source rather than amino acids, yeast extract, peptone, tryptone, were added at a concentration of 0.5 % (w/v) to the fermentation medium broth, keeping other ingredients constant (Amena et al. 2010). Inorganic nitrogen sources were not tried as it would interfere in enzyme assay. In another study L-asparagine as the best amino acid, was added in different concentrations, 0.25, 0.5, 0.75 and 1.25% (w/v). Tryptone was the best nitrogen source other than amino acids, was used in different concentrations varied from 0.25, 0.5, 0.75, 1.0 and 1.25% (w/v), and 0.1% (w/v) of L-asparaginase was added.

The inoculum size: The effect of inoculum size on the production of L-asparaginase was studied, four different size (10 ml, 20ml, 30ml and 40ml/100ml medium) of inoculum was examined. After inoculation, the flasks were incubated at 35±2°C on a shaking incubator (150rpm) and the Free-Cell Filtrate (FCF) assayed for ASNase accumulation after 72h.

RESULTS

Screening of L-asparaginase production of isolated bacteria by plates: The primary screening was carried out using phenolred as pH indicator. Colonies with pink zone were selected as asparagine degrading bacteria. After 72h incubation at 35±2°C. Six bacterial isolates of sex marine samples had apinkcolour around its colonies. Isolates showed a great ability to change the color of the medium to pink in all incubation periods Table1 and Fig.1.

Molecular characterization of bacterial isolate based on 16S rDNA: DNA sequences analyzed using Nucleotide BLAS Talignement tools identified the highest L-asparaginase production isolate. It was identified as H. alkaliantarctica. The sequence submitted into the Bacterial or Archaeal 16S ribosomal RNA sequences database under accession number MK072693.

Optimization the production of L-asparagenase by Halomonas alkaliantarctica: The highest production of the enzyme 59.48 U/min/ ml, when the culture of H. alkaliantarctica was incubated in shaking incubator after 72h at35±2°C at 150rpm, while under static conditions it was 13.79 U/min/ ml. The isolate produced different amount of the enzyme in the presence of different carbon sources, but compared with control, the amount of the enzyme was higher in the presence of asparagine as sole organic source, 70.69 U/min/ml Fig.3.

Results in Fig.4 revealed that, presence ofL-asparaginate as the sole nitrogen and carbon source revealed to the highest amount of the enzyme, 106.09 U/min/ml while glycine was the best second source, 102.16 U/min/ml compared with control. Further, it was equal in the presence of with or without methionine by H. alkaliantarctica. Increasing of L-asparaginate as nitrogen source in the fermentation medium increased the production ofASNasein the fermentation medium, the highest amount of ASNase was 98.37 U/min/ml when 1.0% of L-asparaginate was added after 48h of incubation Fig.5. Fermentation medium supplemented with different nitrogen sources rather than amino acids individually. Results in Fig.6 showed the highest ASNase produced, it was 58.32 U/min/ml in the presence of tryptone as an organic nitrogen source. Results in Fig. 7 showed that in the presence of 0.25% (w/v) tryptone mixed with 1.0% (w/v) asparagine maximized ASNase production to 120.66 U/min/ml compared with control. Increasing the inoculum size to 20/100ml medium (v/v) revealed to the highest ASNase production to 126.67U/min/ml. Fig. 8.

DISCUSSION

About six bacterial isolates were obtained from six marine samples from the west coast in Jeddah city, Saudi Arabia, based on the formation of a pink zone around the bacteria as an indication of the ASNase production on the Glucose Asparagin agar (GA agar) plates, supplemented with phenol red (Asselin et al., 1993; Siddalingeshwara and Lingappa, 2010). The present study was to screen and evaluate ASNase activity of the selected bacteria that isolated from different marine samples.
Fig. 1. Screening of L-asparaginase production of ISFC isolated bacterial by plates

Table 1. Screening of L-asparaginase production by isolated bacteria by plates

<table>
<thead>
<tr>
<th>Marine Samples</th>
<th>Sources of bacterial isolates</th>
<th>Bacterial isolates</th>
<th>Qualitative production of L-asparaginase after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>one day</td>
</tr>
<tr>
<td>Soft coral</td>
<td>Sarcopiton sp.</td>
<td>1SFC</td>
<td>+++</td>
</tr>
<tr>
<td>brown alga</td>
<td>Xenia umbellata</td>
<td>2SFC</td>
<td>-</td>
</tr>
<tr>
<td>green alga</td>
<td>Dictyota sp.</td>
<td>1D</td>
<td>+</td>
</tr>
<tr>
<td>solitary disc corals</td>
<td>Ctenactis crassa</td>
<td>1CC</td>
<td>+</td>
</tr>
<tr>
<td>Nerite Snail</td>
<td>Nerita sp.</td>
<td>1N</td>
<td>+</td>
</tr>
<tr>
<td>Bivalvia</td>
<td>Atactodea glabrate</td>
<td>1A</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. Identity percentage of 16S rDNA between the ASNase activity strains isolated from marine samples from different sites in the west coast of Jeddaah in Saudi Arabia and the relative strains in the gene bank.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Name and Accession of No. of the most related isolates</th>
<th>Identity</th>
<th>Coverage</th>
<th>Suggested Name and Accession No. of the isolates obtained in the work.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seq1</td>
<td>Halomonassalkaliantarctica MG456867.1</td>
<td>100%</td>
<td>100%</td>
<td>MK072693</td>
</tr>
</tbody>
</table>

Fig.2. Phylogenetic tree analysis of ASNase activity isolates based on 16S rDNA
Fig. 3. Effect of different carbon sources on L-asparaginase by *Halomonas alkaliantarctica*.

Fig. 4. Effect of different amino acids on L-asparagenase production by *Halomonas alkaliantarctica*.

Fig. 5. Effect of different concentrations of L-asparagine on the production of L-asparagenase by *Halomonas alkaliantarctica*.
Fig. 6: Effect of different nitrogen sources on L-asparaginase production by *Halomonas alkaliantarctica*.

Fig. 7: Effect of different tryptone concentrations on L-asparaginase production in presence of asparagine by *Halomonas alkaliantarctica*.

Fig. 8: Effect of inoculum size on L-asparaginase production by *Halomonas alkaliantarctica*.
**REFERENCES**


******