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AMYLASE FROM MODERATE HALOPHILES ISOLATED FROM WILD ASS EXCRETA

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ABSTRACT

Twenty-four (Mk-1 to Mk-24) isolates were obtained from excreta samples of wild ass after enrichment. Organisms were able to grow optimally at pH 5-6, 30-40˚C temperature and 10-15% NaCl (w/v), no growth below 5% NaCl indicated moderate halophilic nature of isolates. Organisms were Gram's positive, non-capsulated and identified belongs to Bacillus genera on the basis of Bergey's manual. Nine moderate halophiles were able to produce extracellular amylase, Bacillus macquariensis was the best amylase producer. The organism secretes maximum amylase at pH 6, Temperature 30˚C, 1.2% starch and peptone as nitrogen source after 72 hours.

KEYWORDS: Halophilic amylases, Moderate halophiles, Rann of Kutch, Wild ass.

1. INTRODUCTION

The wild Ass sanctuary is located in the Little Rann of Kutch, Gujarat, India. It covers an area of 4954 km². The sanctuary is named after a wild ass (*Equus hemionus khur*), an animal that inhabitant of sanctuary and could tolerate 48˚C or more temperature and long period of drought. Little Rann of Kutch is a typical ecological system with saline desert climate having unique floral and faunal diversity.

Nature has created extremophilic organisms that could grow and tolerate extreme conditions. Halophiles are the group of extremophiles that requires NaCl for growth, contrary halotolerant microbes doesn't require NaCl[1]. Hydrolytic enzymes such as lipases, amylases, proteases, chitinases, DNases, xylanases, pullulanases etc. have its wide scale applications in food, feed, chemical and pharmaceutical industries [2, 3, 4, 5].

Halophiles produce salt and thermo tolerant hydrolytic enzymes with great industrial potential. α-Amylase (E.C.3.2.1.1) are the hydrolytic enzymes that can catalyze hydrolysis of α 1-4 glycosidic bond in starch and converts it to low molecular weight products such as maltose, glucose, maltotriose units [6, 7, 8]. Amylases have great significant in commercial market with approximately 25% of worlds total enzyme market[9]. Amylases are widely used in baking, brewing, detergent, textile, paper, distilling and pharmaceutical industries[10].

2. MATERIALS AND METHODS

2.1 Collection of samples

Samples (Excreta of wild ass) were collected from little Rann of Kutch, from wild ass sanctuary near Dhrangadhra, Gujarat, India [Latitude- 22°98'4.181"N and Longitude- 71°51'0.242"E].

2.2 Enrichment and isolation of halophiles

Halophiles were enriched in halophilic broth (Himedia) containing (gm/lit); Casein acid hydrolysate-10, Yeast extract- 10, Protease peptone-5, Trisodium citrate- 3, Potassium chloride- 2, Magnesium sulfate- 25, Sodium chloride- 50-150, pH- 7.0-7.4 as well as complete media broth containing (gm/lit); Glucose- 10, Potassium dihydrogen phosphate- 10, Yeast extract- 5, Peptone- 5, Sodium chloride- 50-150, pH- 7.0-7.4. From enriched 15% NaCl (w/v) halophilic broth and complete media broth organisms were streaked on respective agar media by four sector method for the purpose of isolation into pure culture. Total 24 isolates were obtained and preserved on N-agar slant at 4˚C for further studies.

2.3 Morphological and biochemical characterization

Morphological characterization of moderate halophiles was performed by Gram's staining and capsules staining. Biochemically isolates were analyzed on the basis of Bergey's Manual of Systematic Bacteriology.

2.4 Screening of extracellular amylase producers

Amylase producers were determined by method described earlier [11] on starch agar containing (gm/ 100ml); Starch- 0.2, Yeast extract- 0.5, Peptone- 1, NaCl- 10, Agar- 3 and pH- 7.2. After incubation for 2 days at 30°C, iodine solution (gm/100ml; Iodine- 0.33, KI- 0.66) was added. Colonies showing clear zone surrounding against blue background was taken as an evidence of amylolytic activity.

2.5 Amylase assay

Enzyme assay for amylase was carried out by Dinitrosalicylic acid (DNSA) method using starch as a substrate. 0.5ml enzyme was added to 0.5ml 1% starch solution. The reaction mixture was incubated at 37° C for 10 min and then the enzyme reaction was terminated by the addition of 1.0 ml Dinitrosalycilic acid reagent (1g, Dinitrosalicylic acid; 1.6 g, NaOH; 30g, Sodium potassium tartarate; 100ml, D/W). After termination, the reaction was kept in boiling water bath for 10 min. The reaction mixture was diluted up to 10 ml by addition of D/W and the absorbance was measured at 540 nm. One unit of amylase was defined as the amount of enzyme liberating 1 µg of maltose per minute under the assay conditions. Enzyme units were measured using standard maltose (100-1000 µg).

2.6 Optimization of media and environmental conditions

2.6.1 Effect of pH on growth and amylase production

Starch broth (Starch- 0.5, Yeast extract- 0.5, Peptone- 1, NaCl- 10, and D/W- 100 ml) with pH-5, 6, 7, 8, and 9 was inoculated with activated culture of *Bacillus macquariensis* followed by incubation at 30° C in shaking condition. Biomass (O.D. at 540 nm) and enzyme activity were measured after 72 hrs.

2.6.2 Effect of temperature on growth and amylase production

Starch broth with pH 6 was inoculated with fresh culture of *Bacillus macquariensis.* Flasks were incubated at different temperature i.e. 20°C, 30°C, 40°C, 50°C and 60°C followed by measurement of biomass and enzyme activity after 72hrs.

2.6.3 Effect of starch concentration on growth and amylase production

Activated bacterial culture was inoculated in starch broth containing (gm/100ml) Yeast extract- 0.5, NaCl- 10, peptone- 1, pH-6, starch- 0.3%, 0.6%, 0.9% and 1.2%. The flasks were incubated at 30°C, biomass and enzyme activity were measured after 72 hrs.

2.6.4 Effect of nitrogen sources on growth and amylase production

Activated culture of bacteria was inoculated in starch broth with pH 6 and different nitrogen sources like Peptone, glycine, ammonium sulfate and urea at the concentration of 1 % (w/v). The flasks were incubated at 30°C, biomass and enzyme activity were measured after 72 hrs.

3. RESULTS AND DISCUSSION

3.1 Isolation

Samples (wild ass excreta) collected from wild ass sanctuary were weighted 10-15 gram, blackish and dry in appearance. Organisms were enriched in halophilic broth and complete media broth by increasing salt concentration followed by isolation in pure culture on respective agar media. Altogether, 24 isolates were obtained that grow could optimally at 10-15% NaCl, not below 5% NaCl (w/v), indicated moderate halophilic nature. *Halobacillus* sp. strain MA-2, moderate halophile, has similar salt tolerance properties [12]. Such a high salt tolerance by intestinal microbial flora is unusual. This might due to salty grass that utilizes by wild ass as a food. Additionally, all could tolerate bile salt, indicated truly intestinal origination.

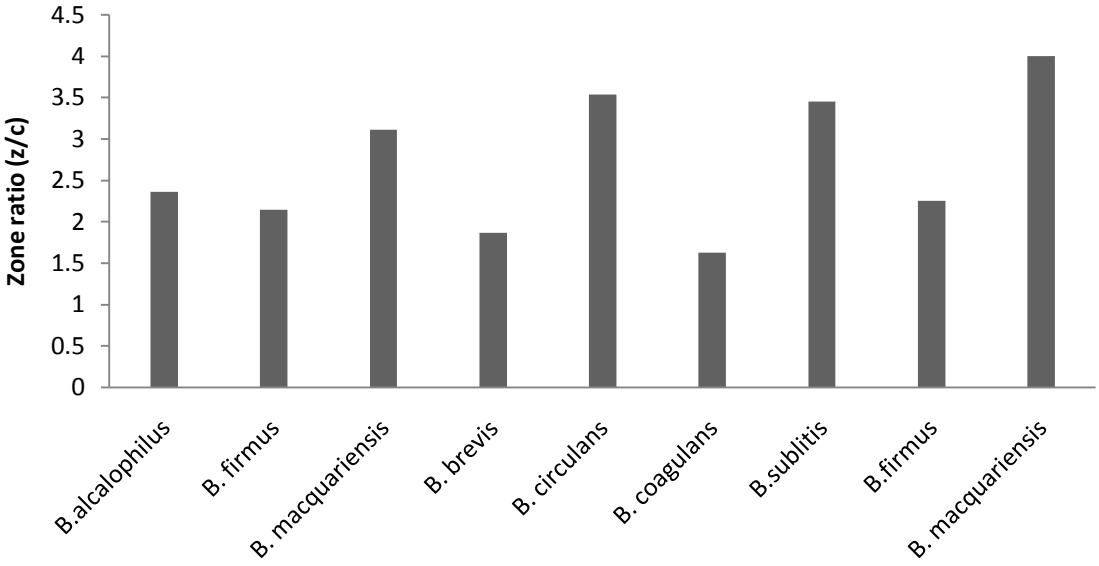
3.2 Characterization

All the isolates were Gram's positive and non-capsulated. Biochemically, all the isolates were belonging to *Bacillus* genera, as revealed by Bergey's Manual of Systematic Bacteriology. Majority of organisms were not able to use urea, tryptophan and citrate, similar results were also displayed by previously isolated halotolerant *Bacillus* sp. [13]. None of the isolates could grow on EMB and MacConkey's agar, indicated colonization of Gram positive organisms in wild ass intestine and elimination of Gram negative one. All the organisms were able to produce catalase, indicated aerobic nature of the isolates. Organisms were highly diversified for sugar utilization such as glucose, xylose, fructose, maltose, sucrose etc.

3.3 Screening of amylase producers

Amylase producers were screened on medium containing starch as an inducer. Nine isolates were secreting amylase. On the basis of zone ratio on solid media, *Bacillus macquariensis* was found to be a potent amylase producer (**Figure-1**).

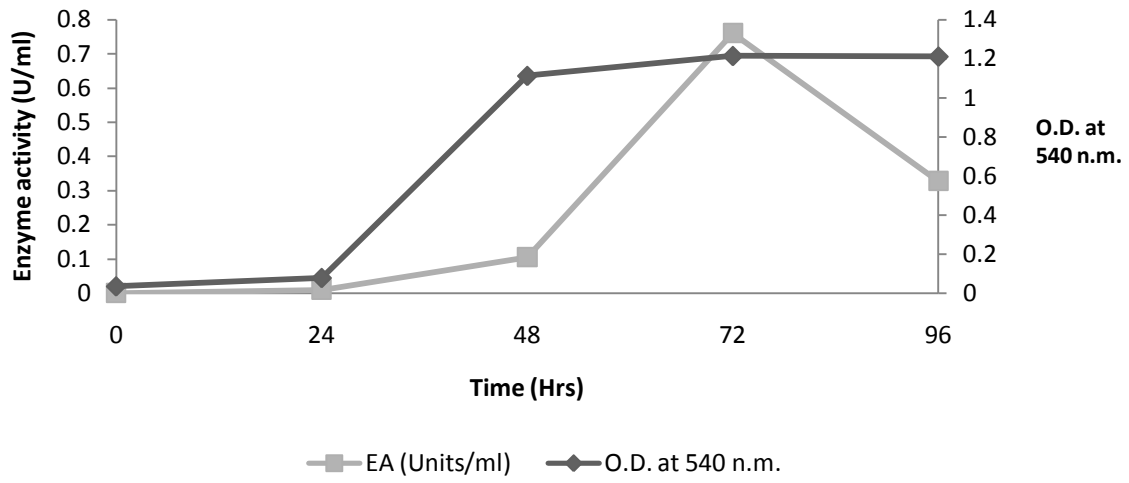
Figure-1 Zone ratio of isolates on starch agar



3.4 Growth kinetics with reference to amylase production

B. macquariensis produces maximum amylase after 72 hours of incubation. Organism has ability to produce amylase in stationary phase (**Figure-2**). *Halomonas meridiana,* moderate halophilic bacteria produces amylase in the absence of glucose until the end of the exponential phase [14].

Figure-2 Growth kinetics and amylase production from B. macquariensis



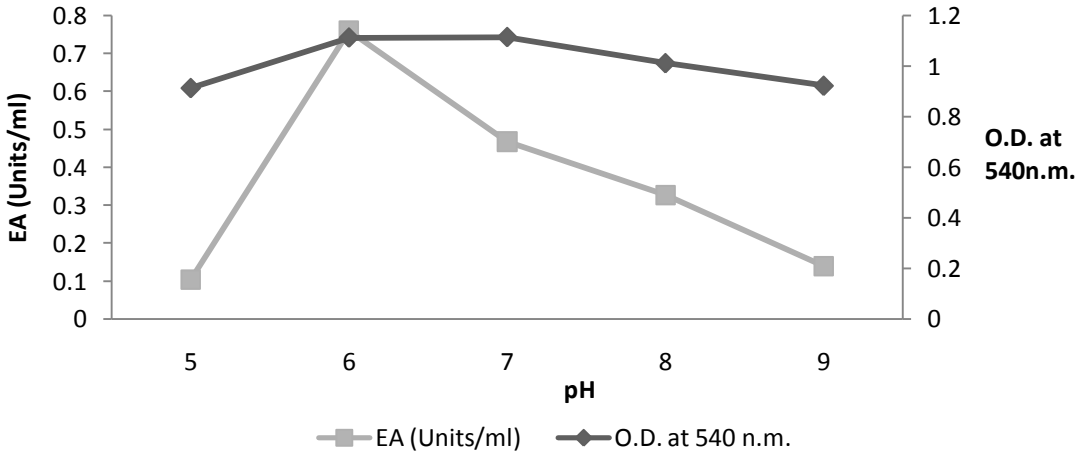
3.5 Media optimization

Optimizations of physical and chemical parameters are important at industrial scale to cope up with economy of the process [15].

3.5.1 Effect of pH on growth and amylase secretion

Amylase production from *B. macquariensis* was maximum at pH 6. Increase or decrease in pH largely affects amylase secretion (**Figure-3**). *B. amyloliquefaciens* and *B. licheniformis* requires pH 7.0 for maximal amylase production[16, 17].

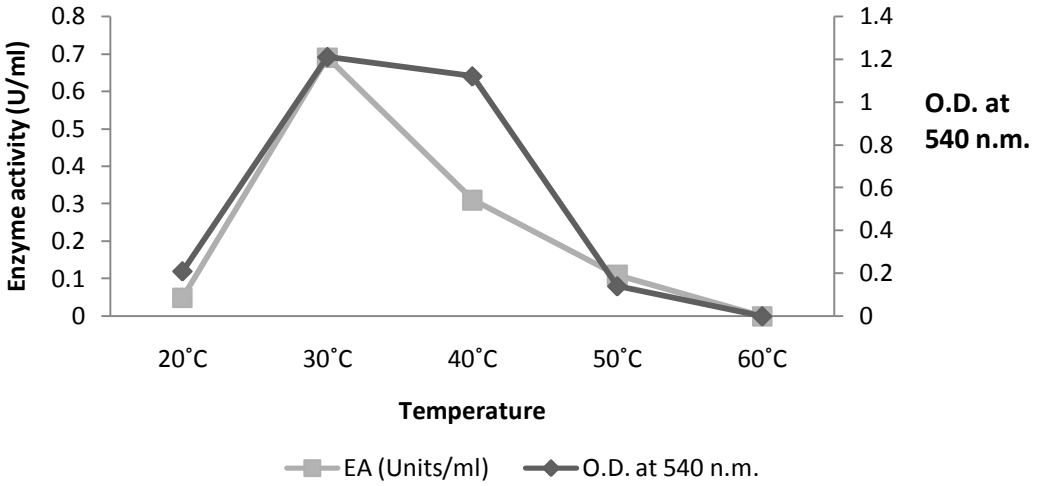
Figure-3 Effect of pH on growth and enzyme production from *B. macquariensis*



3.5.2 Effect of temperature on growth and amylase secretion

B. macquariensis produces maximum amylase and biomass at 30˚C. Lower (20˚C) and higher temperature (50-60˚ C) reduces or prevents growth as well as enzyme secretion (**Figure-4**). Similar results were also obtained from amylase from *Bacillus amyloliquefaciens* [18].

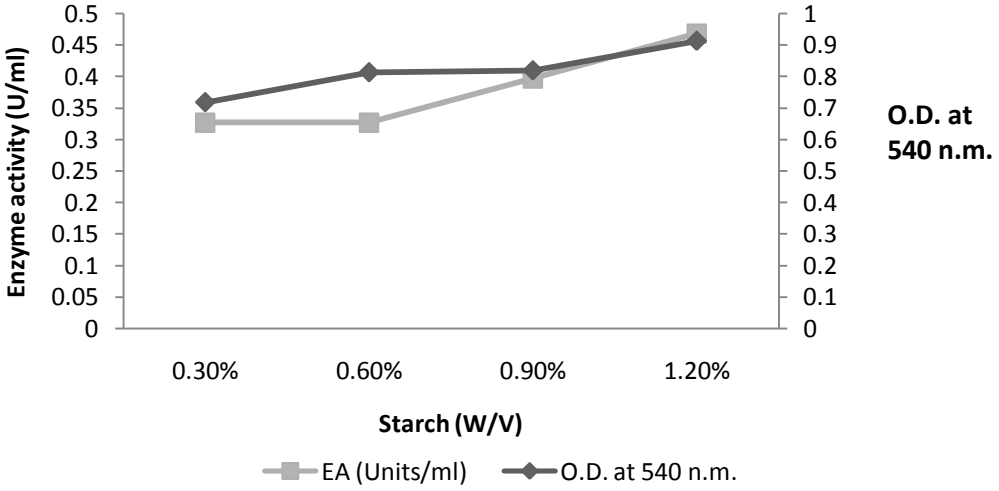
Figure-4 Effect of temperature on growth and amylase secretion from B. macquariensis



3.5.3 Effect of substrate on growth and amylase secretion

Data indicates that growth and enzyme production increases as substrate concentration increases. Optimum growth and enzyme production was achieved at 1.2% (w/v) starch (**Figure-5**). *Halobacillus* sp. strain MA-2, a moderately halophilic bacteria, secrete maximum amylase in the presence of maltose followed by starch[12].

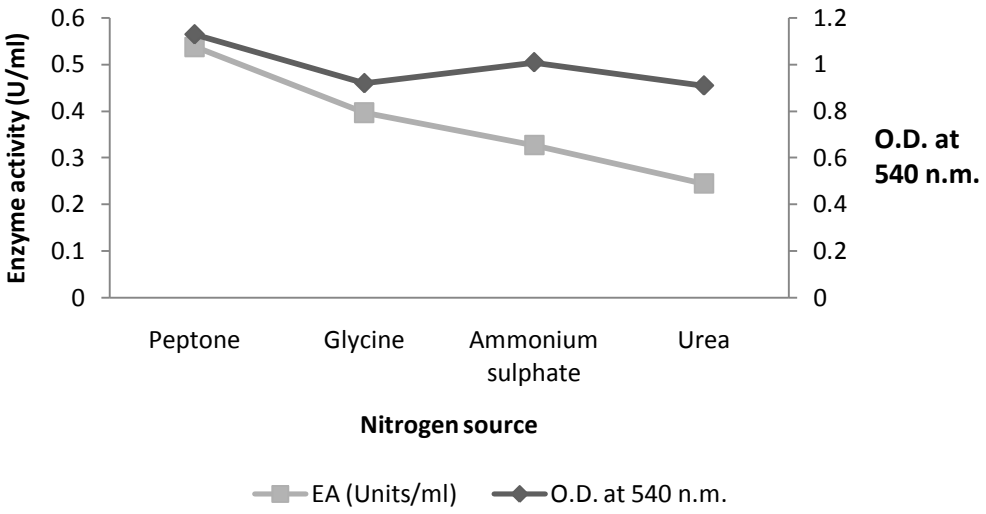
Figure-5 Effect of substrate on growth and amylase secretion from B. macquariensis



3.5.4 Effect of nitrogen sources on growth and amylase secretion

Different organic and inorganic nitrogen sources were incorporated into medium at 1% concentration. Peptone is the nitrogen source that was proved to be optimum for growth and enzyme production, followed by glycine, ammonium sulfate and urea (**Figure-6**). A report exists on *Bacillus megaterium,* indicated peptone as nitrogen source increases amylase secretion [19].

Figure-6 Effect of nitrogen sources on growth and amylase secretion from *B. macquariensis*



4. CONCLUSION

Amylases are used in brewing, backing, pharmaceutical and food industries and plays important role in industrial processes. Fungi and bacteria are used for the production of amylases industrially but halophilic microbe produces salt and thermotolerant amylases. Our isolates were derived from comparatively unexplored site i.e. wild ass excreta.

5. REFERENCES

1. Garabito M.J., Maquez M.C., Ventosa A. Halotolerant *Bacillus* diversity in hypersaline environments. Can J Microbiol, 44: 95-102, (1998).
2. Rao M.B., Tanksale A.M., Ghatge M.S., Deshpande V.V. Molecular and Biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev, 62: 597–635, (1998).
3. Pandey A., Benjamin S., Soccol C.R., Nigam P., Krieger N., Soccol V.T. The realm of microbial lipases in biotechnology. Biotech Appl Biochem, 29: 119–131, (1999).
4. Kulkarni N., Shendye A., Rao M. Molecular and biotechnological aspects of xylanases. FEMS Microbiol, 23: 411–456, (1999).
5. Sanchez-porro C., Martin S., Mellado E., Ventosa A. Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. J Appl Microbiol, 94: 295–300, (2003).
6. Gupta R., Gigras P., Mohapatra H., Goswami V.K., Chauhan B. Microbial α-amylases: a biotechnological perspective. Process Biochem, 38: 1599 – 1616, (2003).
7. Kandra L., α-Amylases of medical and industrial importance. J Mol Stru, 666: 487–498, (2003).
8. Rajagopalan G., Krishnan C. Alpha-amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. Bioresour Technol, 99: 3044-3050, (2008).
9. Reddy N.S., Nimmagadda A., Sambasiva Rao K.R.S. An overview of the microbial α-amylase family. Afr J Biotechnol, 2: 645- 648, (2003).
10. Asgher M., Asad M.J., Rehman S.U., Legge R.L. A thermostable alpha amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. J Food Eng, 79: 950-955, (2007).
11. Amoozegar M.A., Salehghamari E., Khajeh K., Kabiri M., Naddaf S. Production of an extracellular thermohalophilic lipase from a moderately halophilic bacterium, *Salinivibrio* sp. strain SA-2. J basic Microbiol, 48: 160–167, (2008).
12. Amoozegar M.A., Malekzadeh F., Khursheed A.M. Production of amylase by newly isolated moderate halophile, *Halobacillus* sp. strain MA-2. J Microbiol Methods, 52: 353-359, (2003).
13. Muntyan M.S., Tourova T.P., Lysenko A.M., Kolganova T.V., Fritze D., Skulachev V.P. Molecular identification of alkaliphilic and halotolerant strain *Bacillus* sp. FTU as *Bacillus pseudofirmus* FTU. Extremophiles, 6: 195- 199, (2002).
14. Coronado M., Vargas C., Hofemeister J., Ventosa A., Nieto J.J. Production and biochemical characterization of an alpha-amylase from the moderate halophile *Halomonas meridian*. FEMS Microbiol, 183: 67-71, (2000).
15. Francis F., Sabu, A., Nampoothiri K.M., Ramachandran S., Ghosh, S., Szakacs G., Pandey A. Use of response surface methodology for optimizing process parameters for the production of α-amylase by *Aspergillus oryzae*. J Biochem Eng, 15: 107–115, (2003).
16. Haq I., Ashraf H., Qadeer M.A., Iqbal J. Pearl millet a source of alpha amylase production by *Bacillus licheniformis*. Bioresour Technol, 96: 1201-1204, (2005).

17. Syu M.J., Chen Y.H. A study on the α-amylase fermentation performed by *Bacillus amyloliquefaciens*. Chem Eng J, 65: 237-247, (1997).
18. Tanyildizi M.S., Ozer D., Elibol M. Production of bacterial α- amylase by *B. amyloliquefaciens* under solid substrate fermentation. Biochem Eng J, 37: 294–297, (2007).
19. Gurudeeban S., Satyavani K., Ramanathan T. Production of Extra Cellular α-amylase using *Bacillus megaterium* isolated from White Mangrove (*Avicennia marina).* Asian J Biotechnol, 3: 310-316, (2011).

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