Volume : 5 | Issue : 12 | December-2016 • ISSN No 2277 - 8179 | IF : 3.508 | IC Value : 78.46

Original Research Paper

Screening, Isolation and Polyphasic Characterization of L-Glutaminase produced from marine *Streptomyces parvus* HSBT0318

Biochemistry

KEYWORDS: : L-Glutaminase, marine actinobacteria, 16S r RNA , screening, characterization.

Hephzibah rani singh — Department of Biotechnology, Gayatri Vidya Parishad College for degree and PG courses (A), Visakhapatnam 530017, Andhra Pradesh, India

Sandeep B.V — Department of Biotechnology, Andhra University, Visakhapatnam 530003, Andhra Pradesh, India

Syamala D — Department of Microbiology, Gayatri Vidya Parishad College for degree and PG courses (A), Visakhapatnam 530017, Andhra Pradesh, India

ABSTRACT

L-glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) is a very important enzyme due to its role as flavor enhancer and antileukemic agent. Salt-tolerant L-glutaminase produced by marine bacteria is favorable in food industries. This study describes the screening of Lglutaminase producing marine actinobacteria along the coast of the Bay of Bengal, Andhra Pradesh, India. Screening of L-glutaminase was performed using a minimal glutamine medium and identification of selected isolate was performed using molecular-based 16S r RNA gene. Results showed that there were 10 isolates produced positive results of L glutaminase, and one of them (HSBT0318 isolate) produced the highest activity. i.e., 32.12 U/mg, equivalent to the specific activity of 2.48 U/mg. Bacterial identification based on 16S r RNA gene sequencing has revealed that the isolate was 98.9% similar to streptomyces parvus. Characterization of extracellular L-glutaminase from the HSBT0318 isolate showed that the enzyme worked optimally at temperature of 28-55 °C and pH 7. The enzyme was stable when NaCl solution was added up to 13% and began to decrease on addition of NaCl solution of 14% and above with relative activity of 80%. The molecular weight of L-glutaminase was estimated around 45 kDa to 146 kDa.

Introduction :

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells. L-glutaminase (L-glutamine amido hydrolase EC 3.5.1.2) enzyme has attracted significant attention owing to its potential application in medicine as an anticancer agent and could be of significance in enzyme therapy of acute lymphocytic leukaemia. In recent years, L-glutaminase has attracted much attention in both pharmaceutical and food industrial applications. Microbial L-glutaminases are preferred for food and pharmaceutical uses because its production is rapid, inexpensive, gentle, and compatible with downstream steps of its purification (Singh and Banik, 2013).

Marine Actinomycetes are the most economically and biotechnologically significant prokaryotes having the competence to produce many different biologically active secondary metabolites such as vitamins, antibiotics, pesticides and cosmetics to say about a few. Actinobacteria is a phylum of Gram-positive bacteria with high guanine and cytosine content in their DNA. The G+C content of Actinobacteria can be as high as 70%, though some may have a low G+C content. They can be terrestrial or aquatic. Although understood primarily as soil bacteria, they might be more abundant in freshwaters. Actinobacteria is one of the dominant bacterial phyla and contains one of the largest of bacterial genera, *Streptomyces*. Actinomycetes have evolved as a group with greatest genomic and metabolic diversity. They form a distinct pylogenetic line in the 16S r RNA tree and have been of special biotechnological interest with the discovery of a large number of metabololites produced by its different genera, with different biological activity. This distinct group includes some of the key antibiotic producers, commercially established enzymes and other therapeutically useful compounds.

Materials and Methods

Sample Collection and Pre treatment:

Marine sediment samples of Bay of Bengal along the coast of Andhra Pradesh were screened for isolation of L-Glutaminase producing bacteria over a period of six months. A total of 10 marine sediment samples were collected from different locations of Bay of Bengal along the coast of Andhra Pradesh, from a depth of 30 – 200 meters. These samples were collected using a grab sampler and were aseptically transferred to sterile polythene bags using sterile spatula. The samples were immediately taken to the laboratory and processed for bacteriological analysis. The sediment samples were brown to black in colour and of muddy texture. The collected sediment sample was dried at room temperature for a week. 10 gm of sediment sample was transferred to sterile petriplate and kept at 55°C for 10 minutes. The pretreated sample was used for the isolation of actinomycetes (Pisano *et al.,* 1986).

Selective Isolation of L-glutaminase Producing Actinomycetes:

About 5 gm of sample was taken and suspended in 95 ml of sterile distilled water in a 250 ml conical flask and kept in a rotary shaker with 120 rpm for 30 minutes for the thorough mixing of the sediment sample. About 1 ml of mixed sediment suspension from conical flask was transferred in to 9 ml of sterile distilled water. The sample was serially diluted up to 10 dilutions. Minimal glutamine agar (MGA) medium was prepared and used for the isolation of L-glutaminase producing marine actinomycetes. Components of MGA (gram/litre) include 0.5 KCl; 0.5 MgSo4; 1.0 KH2Po4; 0.1 FeSo4; 0.1 ZnSo4; 25 NaCl; 10 L-glutamine; 0. 012 phenol red in which L-glutamine act as carbon and nitrogen source and phenol red act as pH indicator. After sterilization, the minimal glutamine agar medium was supplemented with two filter sterilized antibiotics *viz*., cycloheximide (20 g/ml) and nalidixic acid (100 g/ml), in order to retard the growth of fungi and bacterial populations (Balagurunathan & Subramanian, 1993). 0.1 ml of aliquot from 10-3 to 10-5 dilutions were taken and spreaded on minimal glutamine agar medium by using sterile L-rod. Plating was done in triplicates. All the plates were incubated at 30°C for 1 month. One uninoculated plate was kept as control. All the plates were observed from second day of incubation.

Selection of L-glutaminase Producing Actinomycetes:

During incubation, morphologically different actinomycete colonies which showed powdery or leathery consistency were selected. To obtain pure culture, the selected colonies were streaked on minimal glutamine agar medium by phase streaking and incubated at 28°C for 7 days. After incubation, morphologically different 10 actinomycete colonies were selected and sub cultured on starch casein agar slants (Balagurunathan, et al. 1991). Both the slant culture and glycerol stock cultures were stored at 4°C until further study. Secondary screening for L-Glutaminase activity was carried out by extra cellular L-asparaginase production in submerged fermentation.The enzyme production medium (EPM) was designed based on the data obtained from the studies conducted for optimization of growth conditions for

Volume : 5 | Issue : 12 | December-2016 • ISSN No 2277 - 8179 | IF : 3.508 | IC Value : 78.46

maximal enzyme production in MSG broth.

Assay of L- Glutaminase:

L-Glutaminase activity was determined using L-Glutamine as substrate and the product ammonia, released during the catalysis was measured by using Nesseler's reagent.An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04M L-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml of phosphate buffer (0.1M, pH 8.0). Then the mixture was incubated at 37°C for 15 min and the reaction was arrested by the addition of 0.5 ml of 1.5 M Trichloro Acetic Acid. To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added. The absorbance was measured at 660 nm using a Visible spectrophotometer. One international unit of L-glutaminase was defined as the amount of enzyme that liberates one Mol of ammonia under optimum conditions.

Characterization of selected isolates on the Production of L-glutaminase:

All the isolates were assigned to various genera based on their morphological and biochemical characters outlined in the Bergey's Manual of Systematic Bacteriology (Bucchanan & Gibbons, 1974) . The selected strains of actinomycetes which were used in the later studies were further identified upto their species level based on the schemes suggested in Bergey' s Manual of Systematic Bacteriology (Kreig & Holt , 1984) . Cultural characterization of isolates was studied by inoculating the isolate into standard media for better sporulation. Mature cultures were observed on different media like Tryptone Yeast Extract Agar(ISP1),Tyrosine Agar(ISP7),Yeast Extract Malt Extract Agar(ISP2) and Starch Casien Agar(ISP4). The inoculated plates were incubated for 5 days at 28^{0}C and observed their cultural characteristics.

Polyphasic taxonomic characterization of selected isolates:

Morphological and Cultural characteristics:

Visual observation of both morphological and microscopic characteristics using light microscopy, acid-fastness and Gram-stain properties were performed. All morphological characters were observed on GSM and were used for classification and differentiation.

Molecular characterization

The genomic DNA of the *Streptomyces* strain HSBT0318 was isolated by CTAB lysozyme method. To study the genomic DNA G+C content of the strain by thermal denaturation method (Marmur and Doty, 1962) and for PCR amplification, the actinomycete isolate HSBT0318 was grown on starch casein agar for 4 days at 28˚C.

16S r RNA gene sequencing

Total genomic DNA was isolated using the phenol chloroform method (Nathan et al. 2004). PCR amplification of 16S r-RNA was carried out using the primers FD1 (59-AGAGTTTGATCCTGGG- 39) and RP2 (59-ACGGCTACCTTGTTACGACTT- 39) (Weisburg *et al*., 1991). The PCR product was detected by agarose gel electrophoresis. Sequencing was performed using big dye terminator cycle sequencing kit . The sequence was subjected to homology search using BLAST programme of the National Centre for Biotechnology Information (NCBI) and the sequence data was submitted to the GenBank database under the accession number (Kx907737).

Results and Discussion

Screening and isolation of actinomycetes from marine sediments:

The total count of actinomycetes was isolated in different media. Out of the media used for isolation, starch casein agar (SCA) media and glucose yeast extract malt extract agar (GYM) were favorable for actinomycetes. These results are in agreement with the findings of Kokare *et al*., (2004) who used eight different selective media for isolation of actinomycetes and determined that starch casein agar and glucose yeast extract malt extract agar yielded good growth and more number of actinomycetes than the other types of media.

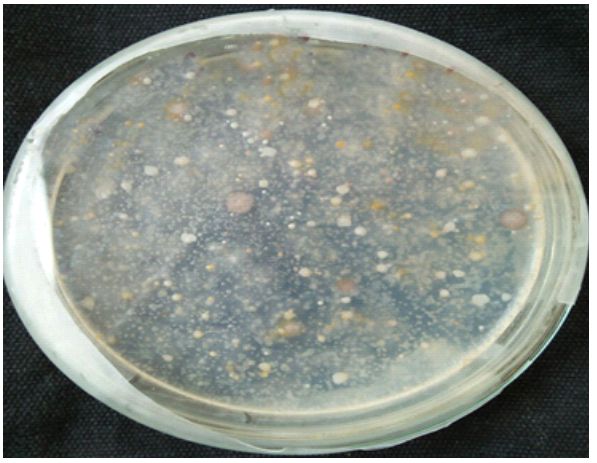

Figure 1: Isolation of marine actinomycetes on SCA Media.

Selection of potential strains for L-glutaminase production was carried out on the basis of quantitative determination of growth and glutaminase production in a mineral salt media supplemented with 1% glutamine as the sole carbon source. Results obtained for the analysis of glutaminase production by l0 strains were individually ranked from l to l0 and HSBT0318 showed the highest activity thus was taken for further studies (table 1).

Table1: Isolates exhibiting L-Glutaminase activity

Isolates	Relative Enzyme Activity(REA)* (U/ml)
HSBT0101	0.03
HSBT0118	0.16
HSBT0127	1.07
HSBT0179	0.21
HSBT0189	0.68
HSBT0311	1.43
HSBT0313	1.46
HSBT0318	**2.48**
HSBT0323	2.23
HSBT0333	0.40

*REA = Diameter of zone of enzyme activity in mm / Diameter of the colony in mm

From the isolated 10 marine isolates subjected to preliminary screening, only 4 isolates showed potential for L-Glutaminase activity. The potential strain selected after secondary screening were tentatively identified as Streptomyces (HSBT0318), based on their morphological, biochemical and physiological characters according to the schemes outlined in Bergey's Manual of Systematic Bacteriology (Buchanan & Gibbons, 1974: Krieg & Holt, 1984). The isolate **HSBT0318** exhibited the highest specific activity of **2.48** U/mg proteins.

Polyphasic characterization

Morphology and cultural characteristics

The morphological and cultural properties of the selected isolates are shown in **Table 2**. The results revealed that among the 4 isolates, 2 isolates by single at tip of sporophores type, 1 isolates by flexous type and 1 isolate by rectiflexible type of spore chains formation. 2 isolates showed the existence of single spores and the spore formation was not observed in 2 isolates. 1 isolates (HSBT0311) showed black spore mass color, 1 isolate (HSBT0313) showed olive green spore mass color, 1 isolate (HSBT0318) showed dark grey spore mass color and 1 isolate (HSBT0323) showed brown spore mass. The aerial mycelium of 1 isolate, exhibited yellow brown color another 1 isolate HSBT0318 showed grey color aerial mycelium, the

isolate HSBT0323 showed light yellow color, while the aerial mycelium was absent in 1 isolate HSBT0311. The substrate mycelia were well developed, abundant and varied from grey to white color in HSBT0318(**figure 3**) and 2 isolates HSBT0313 and HSBT0323 yellow to grey color . 1 isolate produced dark brown color diffusible pigment, (HSBT0311). 1 isolate produced bluish green color diffusible pigment, (HSBT0313), 1 isolate produced greenish brown color diffusible pigment, (HSBT0318) and 1 isolate produced reddish brown color diffusible pigment, (HSBT0323).

Table 2: Morphological and Cultural characteristics of the selected isolates

Morphological characteristics	Character	Isolates			
		HSBT0311	HSBT0313	HSBT0318	HSBT0323
	Spore formation	Singly at tip of sporophores	Rectiflexibles	Singly at tip of Sporophores	Flexous
	Spore mass colour	Black	Olive green	Dark Grey	Brown
Cultural characteristics	Growth	Good	Moderate	Good	Good
	Form of the colony	Compact, folded, leathery, raised	Discrete, flat growth raised in centre	Compact raised	Discrete, floccose, powdery
	Aerial mycelium	Absent	Yellow brown	Grey	Light Yellow
	Substrate mycelium	White	Light grey	White	Grey
	Pigmentation in the medium	Dark Brown	Bluish green	Greenish brown	Reddish Brown

Physiological and biochemical characterization

The physiological and biochemical properties of the selected isolates are shown in **Table 4**. Kampfer*et al*., (1991) suggested the physiological tests as indispensible tools for classification and identification of actionomycetes. Melanin pigmentation was shown by isolate HSBT0311 and HSBT0323 in all three media, while isolate HSBT0318 produced melanin in one of the media and isolate HSBT0313 showed no melanin pigmentation . The isolates HSBT0311 and HSBT0313 were H_2S positive while HSBT0318 and HSBT0323 showed negative results. Tyrosine reaction was positive for all the isolates with the exception of HSBT0318. All the isolates exhibited the hydrolysis of starch and casein hydrolysis.

The capability for nitrate reduction was absent in isolate HSBT0311, and gelatin liquefaction ability was also absent in isolate HSBT0311. Whereas the ability for nitrate reduction and gelatin liquefaction was present in the other 3 isolates (HSBT0313, HSBT0318 and HSBT0323). All the isolates showed good growth in the pH range of 6-9. Isolates HSBT0311, HSBT0318 and HSBT0323 showed good growth in alkaline pH of 7-8. All the isolates grew well at 28°C and the temperature range of all the isolates for growth was from 15 to 37°C. These results are in accordance with Goodfellow and Willams (1983), who reported that most of the actinomycetes behave as mesophiles with an optimum growth at 30°C. All the isolates exhibited salt tolerance in the range of 7-8%, indicating that the isolates are indigenous to marine environment (Haritha *et al*., 2010). The cell wall peptidoglycan of 4 isolates (HSBT0311, HSBT0313, HSBT0318 and HSBT0323) contained LL diaminopimelic acid and traces of glycine and no diagnostic sugars were detected in whole-cell hydrolysates, indicating that it was of cell-wall chemotype I (Lechevalier&Lechevalier, 1970a), which is a salient feature of the genus *Streptomyces*.

Table3: Physiological and Biochemical characteristics of selected isolates

Character	Isolates			
	HSBT0311	HSBT0313	**HSBT0318**	HSBT0323
Grams Staining	+	+	+	+
Melanin Production	+	--	--	+
a. ISP-1	+	--	--	+
b. ISP-6	+	--	+	+
c. ISP-7				
H_2S production	+	+	--	--
a. ISP-6				
Tyrosine reaction	+	+	--	+
a. ISP-7				
Starch hydrolysis	+	+	+	+
Casein hydrolysis	+	+	+	+
Gelatin hydrolysis	--	+	+	+
Nitrate Reduction	--	+	+	+
Methylred Test	--	--	--	--
Citrate Test	+	+	--	+
Catalase test	+	--	+	+
Urea test	+	+	--	+
Growth temperature	--	--	--	--
a. 10°C	+	+	+	+
b. 20°C	+	+	+	+
c. 28°C	+	+	+	+
d. 37°C				
Maximum NaCl tolerance	8%	7%	8%	7%
pH tolerance	6-8	7-9	6-8	6-8
Cell Wall Type	I	I	I	I

+: Positive; --: Negative

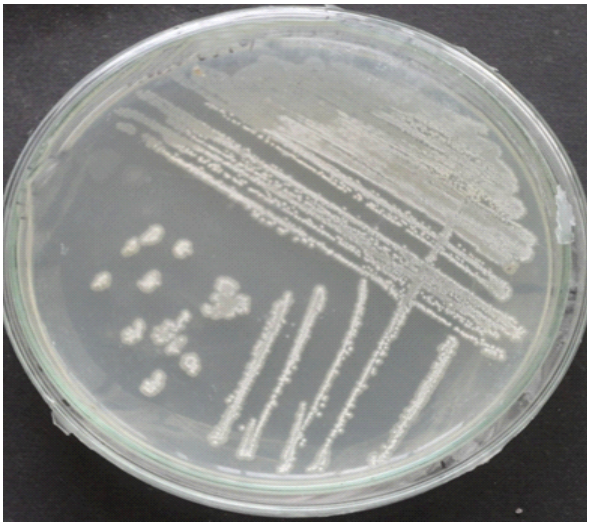

Figure 2: Pure culture of HSBT0318identified as Streptomyces sp.exhibiting white color mycelium with colorless secretions

Volume : 5 | Issue : 12 | December-2016 • ISSN No 2277 - 8179 | IF : 3.508 | IC Value : 78.46

Molecular characterization

The G + C content of the DNA of the strain HSBT0318 was determined as 61%. A BLAST search of the GenBank database using the 716 bp 16S rRNA gene sequence of strain HSBT0318 showed its similarity to that of many species of the genus Streptomyces parvus.

Conclusion

Marine sediment samples of Bay of Bengal along the coast of Andhra Pradesh were screened for isolation of L-Glutaminase producing bacteria over a period of six months. A total of 10 marine sediment samples were collected from different locations of Bay of Bengal along the coast of Andhra Pradesh, from a depth of 30 – 200 meters. Identification using 16S r RNA sequencing revealed the isolate has 98.9% similarity to streptomyces parvus strain JX013965. L-glutaminase has been produced maximally in fermentation condition of 30 °C, 120 rpm, pH of media 7,0, and with starter inoculum of 5%. The crude enzyme can perform optimally at 37- 45 °C, pH 7.0 and retain 50% relative activity when added with NaCl up to 8 . Result of SDS-PAGE revealed that the L-glutaminase might approximately had molecular mass of 45 and 146 kDa.

References:

1. Abdallah, N.A., Amer S.K., and Habeeb, M.K. 2012. Screening of L-Glutaminase Produced By Actinomycetes Isolated From Different Soils In Egypt. International Journal of ChemTech Research. 4(4): 1451-1460.
2. Al Hammed, A.M.A. and Jassim, I.M. 2011. Effect of the antibiotics on Glutaminase purified from Serratia plymuthica isolated from diyala rive. Diyala Agric. Sci. Journal. 3(1): 22-33.
3. Balagurunathan, R., Radhakrisman, M., and Somasundaram, S.T. 2010. L-glutaminase
4. Producing Actinomycetes from Marine Sediments- Selective Isolation, Semi Quantitative Assay and Characterization of Potential Strain. Australian Journal of Basic and Applied Sciences. 4(5): 698-705.
5. Buchanan, RE., Gibbons, NE. (1974). Bergey's manual of determinative Bacteriology, 8th ed., The Williams and Wilkins co, Baltimore, U.S.A.
6. Bergey, DH. (1989). Bergey's Manual of Systematic Bacteriology, Vol. 4. Baltimore, USA: Williams & Wilkins Company. ISBN 0-683-09061-5.
7. Singh and Banik, Biochemical characterization and antitumor study of L-glutaminase from Bacillus cereus MTCC 1305. Appl Biochem Biotechnol. 2013;171:522–531
8. A B David, R.W. and Craig, B.T. 2010. Glutamine addiction: a new therapeutic target in cancer. USA Trends in Biochemical Sciences. 35: 427–433. Desmond, K. O'toole. 1997. The Role of Microorganisms in Soy Sauce Production. Advances in Applied Microbiology. 45: 87-152.
9. Goodfellow, M., Willams, ST. (1983). Ecology of actinomycetes. Ann Rev Microbiol, 37: 189- 215.
10. Gordon, RE., Barnett, DA., Handerhan, JE., Pang, CHN. (1974). Nocardia coeliaca, Nocardia autotrophica, and the nocardin strain. Int J SystBacteriol, 24: 54–63.
11. Imada, A.I., Nakahama, K., and Isono, M. 1973. Asparaginase and Glutaminase Activities of Microorganism. Journal of General Microbiology. 76: 85- 99
12. Iyer PV, Singhal RS Screening and Selection of Marine Isolate for L-Glutaminase Production and Media Optimization Using Response Surface Methodology. Appl Biochem Biotechnol159: 233–250.2009
13. Kampfer, P., Kroppenstedt, RM., Dott, W. (1991). A numerical classification of the genera Streptomyces and Streptoverticillium using miniaturized physiological tests. J Gen Microbiol, 137: 1831-1891.
14. Kelly, KL. (1964). Inter-Society Color Council – National Bureau of Standards Color Name Charts Illustrated with Centroid Colors. Washington, DC: US Government Printing Office
15. Lechevalier, MP., Lechevalier, H. (1970a). Chemical composition as a criterion in the classification of aerobic actinomycetes. Int J Syst Bacteriol, 20: 435-443.
16. Marmur, J., Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol, 5: 109–118.
17. Meister, A. (1956) Physio1.Rev. Q6: 103
18. Nandakumara, R., Yoshimune, K., Wakayama, M., and Moriguchi, M. 2003. Review Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. Journal of Molecular Catalysis B: Enzymatic. 23: 87–100.
19. Origuchi, M., Sakai, K., Tateyama, R., Furuta, Y., and Wakayama, M. 1994. Isolation and Characterization of Salt-Tolerant Glutaminases from Marine Micrococcus luteus K-3. Journal of Fermentation and Bioengineering. 77(6): 621-625
20. Padma, V., Iyer, and Rekha, S.S. 2009. Screening and Selection of Marine Isolate for L-Glutaminase Production and Media Optimization Using Response Surface Methodology. Appl. Biochem. Biotechnol. 159: 233-250.
21. Prakash, P.J.E., Poorani, P., Anantharaman, and Balasubramaniam, T. 2009. L-glutaminase Production and the Growth of Marine Bacteria. Research Journal of Microbiol. 4: 168-172.
22. Roberts, 1960: Roberts, E. (1960) In the Enzymes (Boyer, P.D., Lardy,H., Myrback, K. eds.),
23. Vo1.IV, Academic Press, New York, 285.
24. Sabu, A. 1999. L-glutaminase Production by Marine Fungi, Thesis. Department of Biotechnology COCHIN University of Science and technology. 682 022 : 168.
25. Shirling & Gottlieb, 1966 Methods for characterization of Streptomyces species. Int. J. Syst. Bacteriol., 16: 313-340.
26. Weisburg, WG., Barns, SM., Pelletier, DA., Lane, DJ. (1991). 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol, 173: 697–703.
27. Xu, P., Li, WJ., Tang, SK., Zhang, YQ., Chen, GZ., Chen, HH., Xu, LH., Jiang, CL. (2005). Naxibacteralkalitolerans gen. nov., sp. nov., a novel member of the family Oxalobacteraceae isolated from China. Int J Syst Evol Microbiol, 55: 1149–1153.
28. Yokotsuka et al., 1987 Pro Pak Asia 87 Conference, Singapore, April 8-10.