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SAMARKAND STATE UNIVERSITY NAMED AFTER SHAROF RASHIDOV

ILMIY AXBOROTNOMA

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ISOLATION, IDENTIFICATION AND OPTIMIZATION OF EXOPOLYSACCHARIDE-BASED BIOFLOCCULANT SYNTHESIS BY SOIL BACTERIA *BACILLUS ATROPHAEUS* NR-12

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Abstract. Bioflocculants are polysaccharide-based, biodegradable macromolecules synthesized by soil microorganisms. A bacterium prodicing an extracellular bioflocculant wasisolated from the salty and dry soil and identified as Bacillus atrophaeus. Effects of culture conditions including carbon sources, nitrogen sources, pH, shaker speed on bioflocculant production were studied. Isolate comparative analysis of 16S rRNA sequence identified the isolate to have 99% similarity to Bacillus atrophaeus and it was deposited in the GenBank as Bacillus atrophaeus NR- 2 with accession number OP679007. The strain was characterized by ability to grow and synthesize the bioflocculant in alkaline conditions – pH 11. It was supposed that the strain can overcome adverse effects of environment – alkalinity and salinity by enhanced production of bioflocculant in stress. In the optimized medium (sucrose and tryptone used as C/N sources) the strain produced 4.76 mL bioflocculant.

Key words: Bioflocculant, Bacillus atrophaeus, soil salinity, media optimization, carbohydrates.

Выделение, идентификация и оптимизация синтеза биофлокулянтов на основе экзополисахаридов почвенными бактериями *bacillus atrophaeus* nr-12

Аннотация. Биофлокулянты представляют собой биоразлагаемые макромолекулы на основе полисахаридов, синтезируемые почвенными микроорганизмами. Бактерия, продуцирующая внеклеточный биофлокулянт, была выделена из соленой и сухой почвы и идентифицирована как Bacillus atrophaeus. Было изучено влияние условий культивирования, включая источники углерода, источники азота, pH, скорость встряхивания, на производство биофлокулянта. Сравнительный анализ последовательности 16S рРНК изолята показал, что изолят имеет 99% сходство с Bacillus atrophaeus, и он был депонирован в GenBank как Bacillus atrophaeus NR-2 с регистрационным номером OP679007. Штамм характеризовался способностью к росту и синтезу биофлокулянта в щелочных условиях – pH 11. Предполагалось, что штамм может преодолевать неблагоприятные воздействия окружающей среды – щелочность и засоленность за счет усиленной продукции биофлокулянта в условиях стресса. В оптимизированной среде (сахароза и триптон, используемые в качестве источников C/N) штамм продуцировал 4,76 мл биофлокулянта.

Ключевые слова: Биофлокулянт, *Bacillus atrophaeus*, засоление почвы, оптимизация сред, углеводы.

Tuproq bakteriyasi bacillus atrophaeus nr-12 ni izolyatsiyasi, identifikatsiyasi va ekzopolisaxarid asosidagi bioflokulyantlar sintezini optimallashtirish

Annotatsiya. Bioflokulyantlar polisakkaridlar asosidagi, tuproq mikroorganizmlari tomonidan sintez qilingan biologik parchalanadigan makromolekulalardir. Hujayradan tashqari bioflokulyantni ishlab chiqaruvchi bakteriya sho'r va quruq tuproqdan ajratib olindi va *Bacillus atrophaeus* ekanligi aniqlandi. O`sish sharoiti, jumladan uglerod manbalari, azot manbalari, pH, chayqatish tezligi bioflokkulyant ishlab chiqarishga ta'siri o'rganildi. Ajratib olingan bakteriyaning 16S rRNK ketma-ketligi *Bacillus atrophaeus* ga 99% o‘xshashligini aniqladi va GenBankda OP679007 kirish raqami bilan *Bacillus atrophaeus* NR-2 sifatida deposit qilindi. Shtamm pH 11 sharoitda bioflokkulyantni o'stirish va sintez qilish qobiliyati bilan ajralib turadi. Shtamm stressda bioflokulyant ishlab chiqarishni kuchaytirish orqali atrof-muhitning salbiy ta'sirini - gidroksidi va sho'rlanishni engishi mumkin deb taxmin qilingan. Optimallashtirilgan muhitda C/N manbalari sifatida saxaroza va tripton ishlatilganda shtamm 4,76 ml bioflokulyant hosil qildi.

Kalit so`zlar. Bioflokulyant, *Bacillus atrophaeus*, tuproq sho'rligi, muhitni optimallashtirish, uglevodlar.

Introduction. Bioflocculant produced by microorganisms have been widely used in a variety of industrial processes, such as wasterwater, drinking water purification and industrial downstream processes [1-3]. Bioflocculant are biopolymer consisting of polysaccharides, polypeptides, monosaccharides, proteins and minerals [4, 5]. Bioflocculants are attracting great research interest due to their safety, biodegradable properties and produce no secondary pollution are sought after [4, 6]. In recent years, the microbial flocculants, their properties and chemical contents has been reported by others researchers. Liu et al. (2010) found novel bioflocculant produced by *Chryseobacterium daeguense* W6, investigated the effects of carbon source, nitrogen source, C/N ratio, initial pH, inoculum size, culture temperature and shaking speed on bioflocculant syntheses [7]. A bioflocculant obtained from the soil bacteria *Bacillus* sp. has been studied effects of physico-chemical conditions including pH, carbon sources and nitrogen sources [1, 8]. Deng et al. (2005) reported *Aspergillus parasiticus* was found to produce a bioflocculant with high flocculating activity. Moreover, found optimal culture condition for production bioflocculant: that the carbon and nitrogen sources favorable for the production of the bioflocculant were corn starch and peptone, and an optimal condition of 28 ◦C, initial pH 5–6 and shaking speed of 150 rpm [9].

In the present study, we investigated the optimal culture condition fof production of a biofluccculant by *B. atrophaeus* NR- 2. Various factors effecting bioflocculant production, such as carbon source, nitrogen source, initial pH, temperature and shaking speed were investigated to determine the optimal culture condition for *B. atrophaeus* NR- 2.

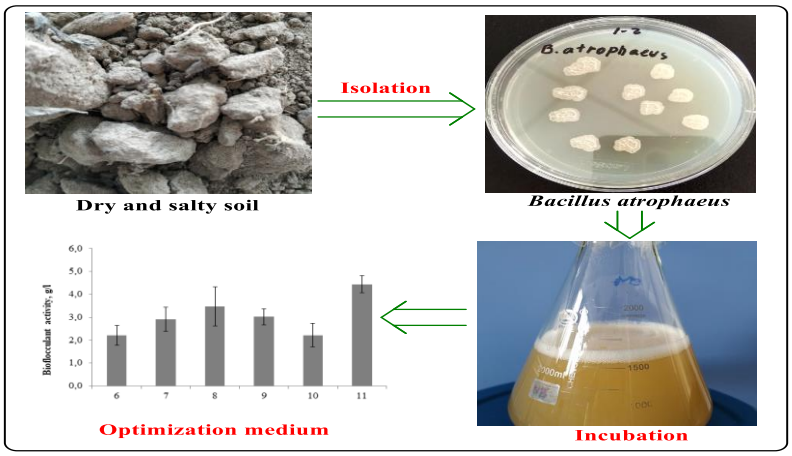


Figure 1. General approach of this research work

Material and methods. Isolation identification of bioflocculant producing bacteria

The soil sample were weighed aseptically (1 g) and mixed with 9 mL sterile phosphate buffered saline (PBS) (150 mM NaCl, 20 mM Na_2HPO_4, pH 7.4) and shaken with a vortex, then serially diluted (10^1–10^5). A 100 µl aliquots from the appropriate dilutions were spread on Yeast Extract Glucose Broth (PYG) (glucose (5 mL), yeast extract (1 mL), peptone (10 mL), NaCl (0.2 mL), K_2HPO_4 (0.2 mL), $MgSO_4$ (0.2 mL, water-1L) and incubated for 3 days at 28˚C. Differentiable colony morphologies were picked from petri dish and re-streaked on PYG medium for the purification of the isolates.

Total 10 purified strains were cultivated in the PYG broth for 3 days. Four milliliters of the culture was transferred into another 250-mL flask containing 100 mL PYG broth and incubated by shaking for 48 h at 30˚C [10, 11]. Subsequently, the culture broth was centrifuged at 10,000 rpm at 4 ˚C for 20 min. Two volumes of cold ethanol were added to the supernatant and white cotton-like flocks were precipitated. The ethanol and the cell-free supernatant mixture were centrifuged at 10,000 rpm for 15 min and the precipitate re-dissolved in distilled water at ratio 1:4 (v/v). The procedure repeated twice and the purified bioflocculant was vacuum-dried to obtain a purified bioflocculant. The isolate NR-12 which showed highest bioflocculant production was taken for further studies.

Identification of strain *Bacillus atrophaeus* NR-12. Soil bacteria *B. atrophaeus* NR-12 was incubated in 250 mL flask containing 50 mL of fresh LB medium for 16 h at 37 ˚C with shaking at 200 rpm. DNA extraction was conducted using E.Z.N.A™ Bacterial DNA Kit (Omega Bio-Tek, USA). PCR amplification was carried out to determine the partial 16S rRNA gene. The PCR thermal cycling scheme was set as follows: 30 cycles of denaturation at 94 ˚C for 1 min, 55 ˚C for 30 s and 72 ˚C for 1.5 min. The

16S universal primers C27F AGAGTTTGATCCTGGCTCAG and C14921R TACGGCTACCTTGTTACGACTT were used to amplify of the 16S rRNA gene. Purification of the PCR products and the determination of sequences were performed by GeneCore BioTechnologies Co, Ltd. (Shanghai, China). The 16SrRNA gene sequence of strain *B. atrophaeus* NR-12 obtained was compared with the NCBI database (htt://blast.ncbi.nlm.nih.gov). The evolutionary history was inferred using the Neighbor-Joining method [12]. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown above the branches [13]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [14] and are in the units of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1490 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [15].

The effect of culture conditions on bioflocculant production. The effect of different carbon and nitrogen sources on bioflocculant production by bacterial isolate was assessed. Carbon sources used in medium included Glucose, Sucrose, Maltose, Mannose, and Fructose (20 mL) [16], while the nitrogen source included ammonium sulphate, ammonium chloride (inorganic nitrogen sources) and urea (organic nitrogen sources). The assessments were done in accordance with previous description of Cosa et al. [17] and procedure of Lachhwani [18].

pH and temperature stability of the biofilocculant. The pH values of 7 groups of sample solutions were adjusted to 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 with NaOH (1 M) and HCl (0.5 M), respectively, and then was assayed flocculating activity of each sample [19]. Each sample was incubated at 25°C, 30°C, 35°C, 40°C, for 48 hour and further determined for their flocculating activity following procedure described in Kothari and Pathak et al. [20].

The bioflocculant extraction. Strain *B. atrophaeus* NR-12 was inoculated into a 50 mL flask containing 10 mL PYG broth and cultivated at 30 °C, 48 h and 150 rpm. Then 4 mL of the culture was added into other 250-mL flask containing 100 mL PYG broth. The bioflacculant was produced by shaking the flask 30 °C, 48 h, 170 rpm. The treated culture broth was centrifuged (1000 rpm, 10 min., 4 °C) and the cell-free supernatant was collected and mixed with three volumes of cold ethanol (95%) and maintained at 4 °C overnight to complete the precipitation of bioflocculant [19, 21].

Results and discussion. Isolation and identification of bioflocculant producing bacteria. Totally 15 bacterial strains were isolated from the soil. Among these strains, strain NR-12 was screened out since the material extracted from its fermentation broth in the screening medium showed the powerful bioflocculant activity. Therefore, we have selected for future study bioflocculant production. The high level production of bioflocculant was derived in strain *B. atrophaeus* NR-12 and identified by 16S rRNA gene sequences method, bacteria 99% similarity to relatives *Bacillus atrophaeus* and deposed in GenBank with accession number OP679007. A phylogenetic tree using the Neighbor-Joining method was constructed, showing the closest relatives of the isolates (Figure 2).

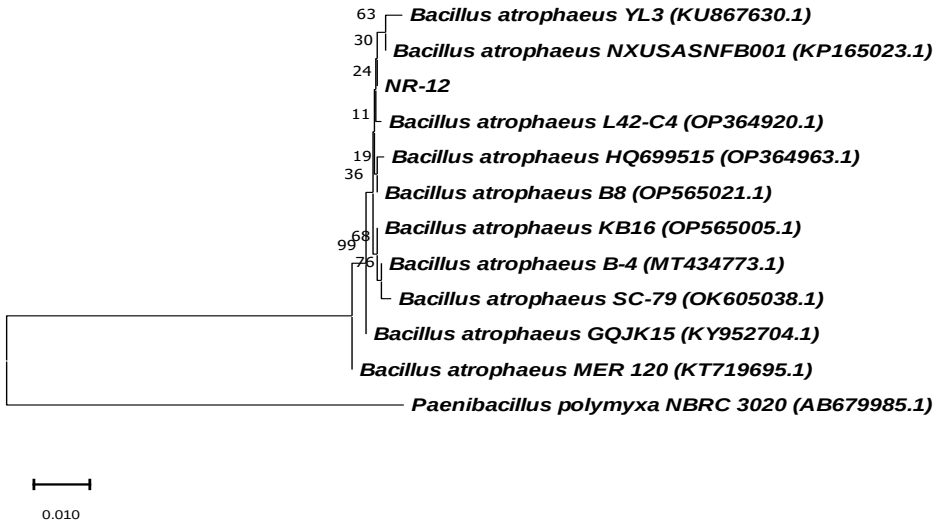


Figure 2. The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences isolated from soil bacteria *B. atrophaeus* NR-12 showing the relation of isolated strains to closest relatives in GenBank.

TGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCT
GTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGT
GGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAACGATG
CGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGT
TGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTT
AAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAG
TGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAA
CTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT
AAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACT
GAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCTCTGACACCCCTAGAGATAGGGCTTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTG
GGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG
GGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAGACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTC
AGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTATG
GAGCCAGCCGCCGAA

Effect of carbon and nitrogen sources on bioflocculant production by *Bacillus atrophaeus* RN-12. The effect of various carbon sources on bioflocculant production by bacteria showed that sucrose was optimal for the production of bioflocculant by selected isolate with bioflocculant activity of 5.6 g/L, followed by glucose with bioflocculant activity 4.1 g/L. The bioflocculant production by bacterial isolate was low under fructose, mannose and maltose (Figure 4 A). The concentration of sucrose (0.01-0.03%) was also effect on the bioflocculant activity of bacterial isolate. The most optimal sucrose concentration was 0.02% (Figure 4 B). The organic nitrogen sources such as yeast extract, malt extract, urea, peptone, L-tryptophan, and tryptone also effect on bioflocculant activity of bacterial isolate. The peptone (3.19 mL), and tryptone (3.63 g/L) resulted in high bioflocculant activity. The optimal concentration of tryptone was 0.8% concentration among other concentrations (0.1-1.0%). The production of bioflocculant by bacterial isolate was 3.63g/L. The low bioflocculant activity was observed under malt extract, L-tyrptophan, and urea (Figure 5).

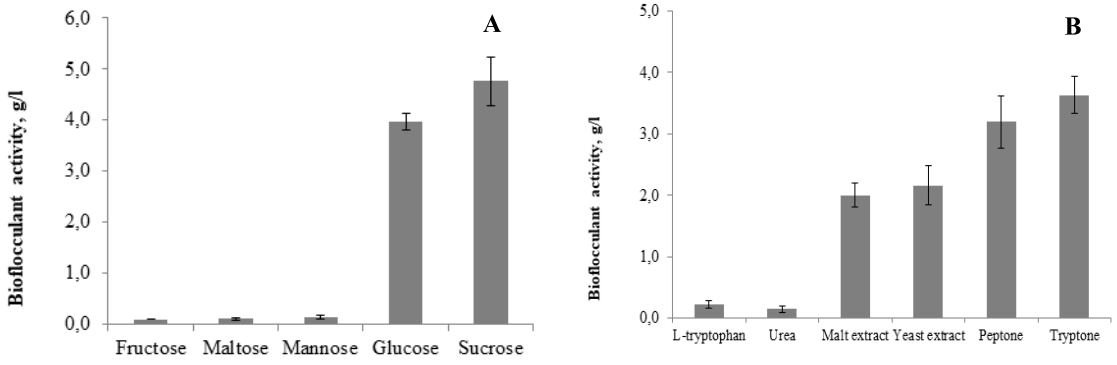


Figure 4. The effect of carbon and nitrogen source on bioflocculant activity of *B.atrophaeus* RN-12

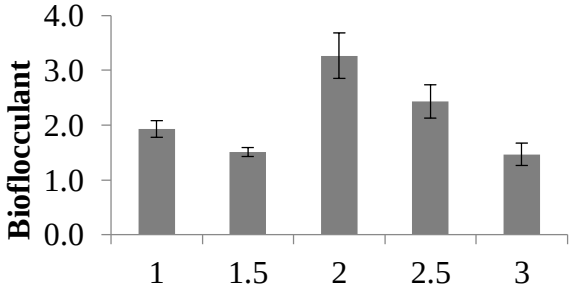


Figure 5. The effect of different concentration of sucrose on the bioflocculant activity of *B.atrophaeus* RN-12

Effect of the initial pH of the fermentation medium and temperature on bioflocculant production. The effect of initial pH of the fermentation medium on bioflocculant production of *B.atrophaeus* RN-12 shown in Figure 6 A. Initial pH of the medium before autoclaving was 7.3 and the culture medium on bioflocculant production was examined at pH values ranging from 5 to 11. The optimum pH for bioflocculant production was 8 and 11 (for 3.43 and 4.43 g/L) respectively (Figure 6 B). The temperature stability of the bioflocculant showed that high effect of bioflocculant production was observed at 40˚C (4g/L).

Effect of culture shaking speed on bioflocculant production and time course

The shaking speed of 170 rpm was the most optimal condition to increase bioflocculant production by bacterial isolate (Figure 7 A). Lower shaking speed caused a decrease in the bioflocculant activity. However, the higher shaking speed than 170 rpm bioflocculnt activity increases but bioflocculants form colloidal solution in the fermentative medium. The time profile of bioflocculant production by the *B. atrophaeus* RN-12 was observed a growth period of 120h (Figure 7 B). During growth the bioflocculanting activity was observed to increase reaching a maximum of 4.13mL at 48h.

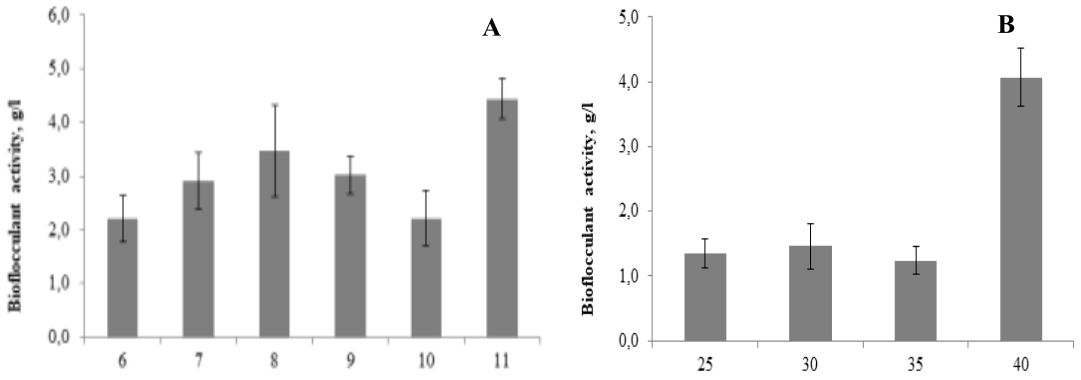


Figure 6. The effect of pH and temperature on the bioflocculant activity of *B. atrophaeus* RN-12

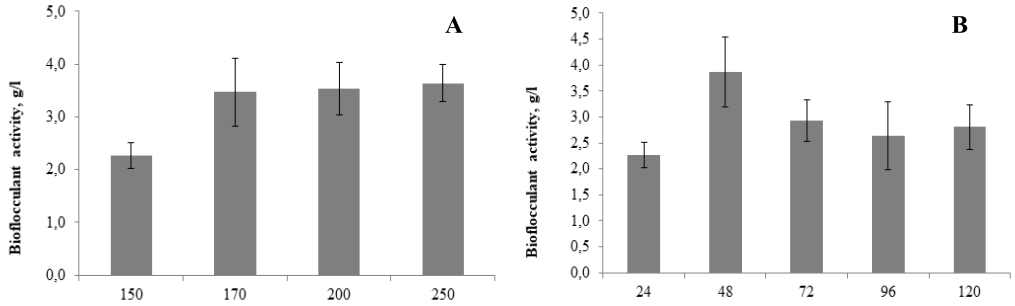


Figure 7 The effect of shaking speed and time course on the bioflocculant activity of *B. atrophaeus* RN-12

GC-MS of the hydrolysate products showed that glucose was the basic carbohydrate and overall carbohydrate portion represented as L-(+)-Rhamnose (0.28%), L-(-)-Fucose (0.20%), D-(+)-Mannose (40.18%), D-(+)-Galactose (3.69%) and Myo-inositol (45.61%).

Table 1. Chemical composition of bioflocculant synthesis by *B. atrophaeus* RN-12

№	Components	RT	Content, %
1	L-(+)-Rhamnose	7.096	0.28
2	L-(-)-Fucose	7.611	0.20
3	D-(+)-Mannose	12.504	40.18
4	D-(+)-Galactose	12.946	3.69
5	Myo-inositol	16.755	45.61

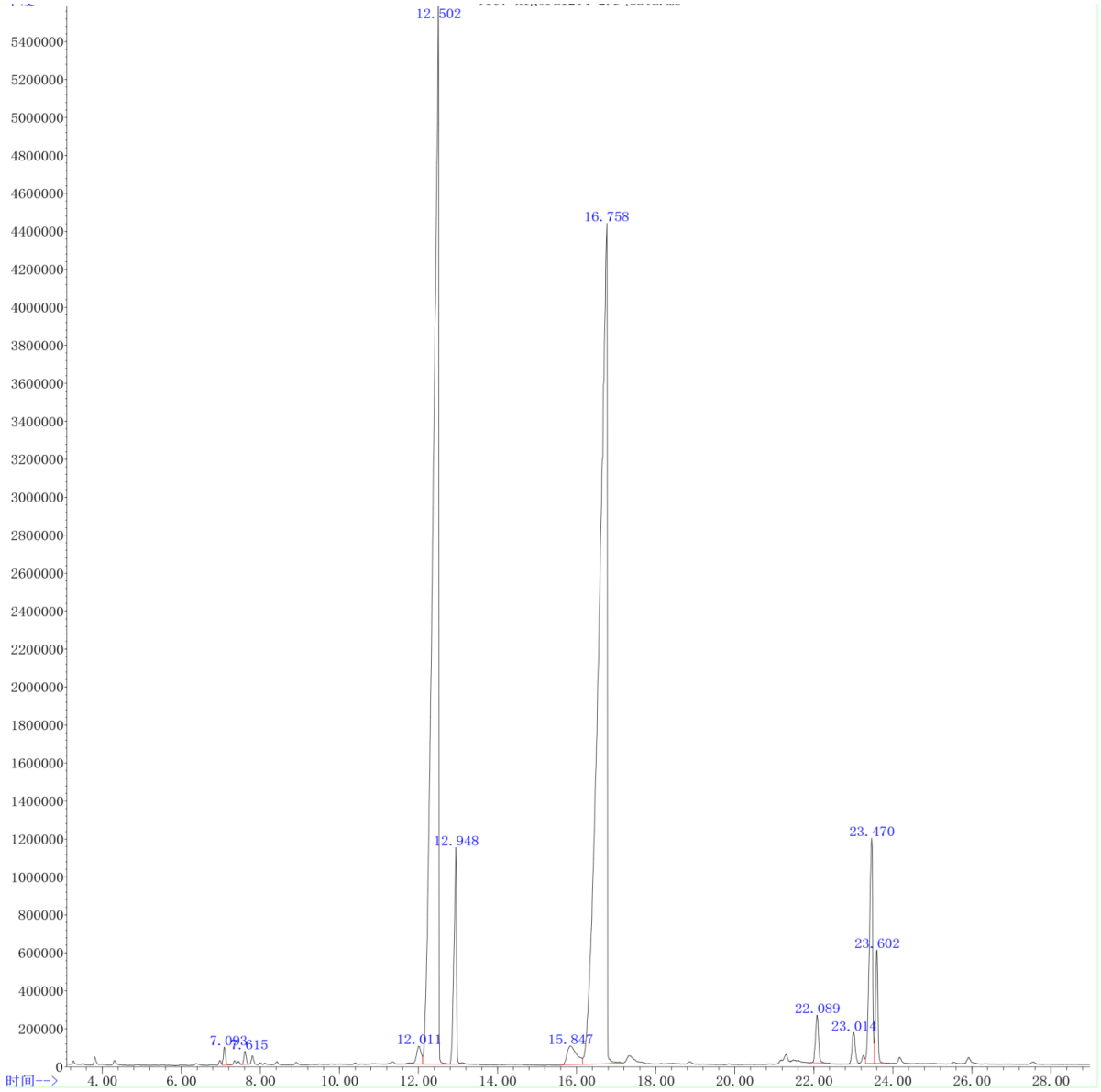


Figure 8. GC-MS analyses of exopolysaccharides-based bioflocculant synthesis by *B. atrophaeus* RN-12

Conclusion. A bioflocculant-producing bacterium *B. atrophaeus* RN-12 was isolated from salty soil and production of the bioflocculant was optimized. Optimal culture condition for production bioflocculant: most optimal carbon of sucrose concentration was 0.02%. The organic nitrogen sources such as yeast extract, malt extract, urea, peptone, L-tryptophan, and tryptone also effect on bioflocculant activity of bacterial isolate. Initial pH 11 and temperature 40 ^{o}C, during growth the bioflocculanting activity was observed to increase reaching a maximum of at 48h and shaking speed 170rpm. Chemical analysis showed that the bioflocculant was a polysaccharide were detected.

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