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# Biosurfactant production by *Pseudomonas aeruginosa* isolated from aquaculture farm soil and its optimisation

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## ABSTRACT

In the present study, aquaculture farm soil was screened for biosurfactant producing bacteria. A total of 43 morphologically distinct colonies were isolated from the farm soil and their biosurfactant production was evaluated employing different screening methods. Fourteen biosurfactant producing bacterial isolates were selected based on the formation of dark blue halos on cetyl trimethyl ammonium bromide (CTAB) agar, emulsification index, oil spreading assay and BATH (Bacterial adhesion to hydrocarbons) assay. Based on the results, the bacterial isolate BHI 9 with highest production of biosurfactant was selected for further studies. The bacterial isolate BHI 9 was found to be Gram negative, slender long rod and oxidase as well as catalase positive. The isolate was identified as *Pseudomonas aeruginosa* based on 16S rDNA sequence analysis. Optimisation studies were carried out at different temperatures (25, 30, 35 and 40°C) using four different carbon sources (1%) *i.e*., glucose, sucrose, maltose and starch and four nitrogen sources (1%) *viz*., peptone, ammonium nitrate, beef extract and yeast extract, at different pH (6, 7, 8, 9 and 10) and NaCl levels (0.5, 1.0, 1.5 and 2.0%). Emulsification index and bacterial biomass ($OD_{600}$) were recorded at 24, 48, 72 and 96 h intervals. Optimum conditions for biosurfactant production by this bacterium was obtained when glucose and yeast extract were used as carbón and nitrogen sources respectively, maintaining a temperature of 35°C, pH 8 and NaCl 1.5%, measured in terms of emulsification index and bacterial biomass. This is the first report on biosurfactant producing bacteria isolated from aquaculture farm soil that can find its application in various fields.

Keywords: Aquaculture, Biosurfactant, Optimisation, *Pseudomonas aeruginosa*, Soil

## Introduction

Biosurfactants are diverse group of biological molecules produced by different kinds of bacteria, fungi and yeast (Nitschke *et al*., 2018). They are heterogeneous type of surface active molecules with distinct physical, chemical and biological properties and have wide range of industrial applications (Satpute *et al*., 2017; Geetha *et al*., 2018; Singh *et al*., 2018). Among different bacteria, Pseudomonads are the best-known bacteria capable of producing biosurfactants by utilising hydrocarbons as carbon and energy sources (Beal and Betts, 2000; Noordman and Janssen, 2002; Saikia *et al*., 2012). Biosurfactants produced by *Pseudomonas* sp. from different sources have been studied by various researchers (Ismail *et al*., 2015; Shekhar *et al*., 2015; Ji *et al*., 2016; Geetanjali *et al*., 2017; Sahebnazar *et al*., 2018). Similarly, from aquatic sources, *Pseudomonas* bacterial strains have been isolated and screened for biosurfactant production potential employing different methods. Balan and Jayalakshmi (2013) isolated *Pseudomonas aeruginosa* from sediment and water samples from Mudasalodai coast and optimised biosurfactant production employing low cost medium. Pepi *et al*. (2013) isolated *P. aeruginosa* DG2a strain from aquaculture waste waters and studied biosurfactant production and fatty acid degradation potential. Gomathy and Senthilkumar (2013) isolated and identified potential oil degrading *P. aeruginosa* from harbour waters of Cuddalore, India. Kiran *et al*. (2014) evaluated glycolipid biosurfactant production by marine bacterium *Brachybacterium paraconglomeratum* MSA21. Antoniou *et al*. (2015) studied the capacity of marine bacterial consortium to produce biosurfactant for degrading hydrocarbons. Sadatfaraji *et al*. (2016) reported on the isolation and identification of biosurfactant producing bacteria from Persian Gulf and evaluated their surfactant activity.

Though much interest is shown towards biosurfactants presently, these do not compete with synthetic surfactants in terms of economics of production. Several studies have been conducted to produce biosurfactants using low cost

production material and employing engineering processes to increase the yield for reducing production cost (Balan and Jayalakshmi, 2013; Banat *et al*., 2014; Satpute *et al*., 2017). Laboratory studies employing microbes need to be conducted for optimisation of biosurfactant production by the microorganisms as it can serve as one of the means for maximum production. Gomathy and Senthilkumar (2013) optimised rhamnolipid type of biosurfactant production by *P. aeruginosa* isolated from marine source. Cost effective technologies for biosurfactant production from renewable substrates was studied by Banat *et al*. (2014). Sadatfaraji *et al*. (2016) studied surface tension of biosurfactant producing bacteria at different temperature, salinity, pH and carbon sources. Sellami *et al*. (2016) optimised agro processing wastes such as molasses and fish processing byproducts for maximum production of biosurfactant by *Aneurinibacillus migulanus*. However, there are no reports available on the isolation and identification of biosurfactant producing bacteria from aquaculture farm soil. Therefore, this study attempted to screen aquaculture farm soil for potent biosurfactant producing bacteria as well as to optimise conditions for biosurfactant production.

## Materials and methods

*Collection of soil sample*

Soil samples employed in this study were collected from shrimp aquaculture farms located in Ernakulam District, Kerala, India (10. 24' N and 76. 2' E). Top soil (about 1 cm) was collected from all the four corners of the farms and pooled to represent the whole farm. Soil samples collected in aseptic sample bags with proper labelling were brought to the laboratory in ice box for further analyses. After reaching the laboratory, the samples were stored in a refrigerator at 4±0.1°C and analysed within 2-4 h of collection. From each soil sample sub-samples were taken for analysis.

*Isolation of biosurfactant producing bacteria*

Bushnell-Hass (BH) medium was used for isolation of biosurfactant producing bacteria (Sahoo *et al*., 2011). Composition of the medium (g $l^{-1}$) is as follows: Magnesium sulphate 0.20; Calcium chloride 0.02; Monopotassium phosphate 1.0; Dipotassium phosphate 1.0; Ammonium nitrate 1.0 and Ferric chloride 0.05. All the ingredients were added and the pH was adjusted to 7.0. For plating, agar (18 g $l^{-1}$) was added to the medium.

*Enrichment and isolation of pure culture*

Approximately 10 g of soil was aseptically weighed and added to BH medium (90 ml) supplemented with 2% diesel and was incubated at 30°C for 96 h. The enriched broth (10 ml) was subsequently transferred to fresh broth with diesel oil for isolating pure culture and incubated under same conditions. Biosurfactant producing bacteria, having raised white creamy colour (2 mm) were isolated by directly plating 0.1 ml of serially diluted enriched samples and spread plated onto the agar plates and incubated at 30°C for 96 h. After incubation, well defined isolated colonies were randomly picked and streaked onto BH medium plate to check for purity. Purified cultures were preserved at -80°C using 60% glycerol until further use.

*Screening of bacterial isolates for biosurfactant production*

*Blue agar plate or CTAB method*

For detection of extracellular biosurfactant production, formation of dark halo zone in the plate added with methylene blue and cetyl trimethylammonium bromide (CTAB) was tested as per Siegmund and Wagner (1991). In BH agar medium supplemented with 1.8% agar, 0.005 g of methylene blue and 0.2 g of CTAB was added and sterilised at 121°C for 15 min. The culture supernatant (10 µl) was added to the plate and kept for incubation for 48 h at 30°C. Formation of dark blue halo around the culture was recorded as an indication of ion pairing between anionic biosurfactant and cationic CTAB-methylene blue agar complex (Saravanan and Vijayakumar, 2012).

*Emulsification index*

Emulsification activity of the bacterial isolates was measured as per the methods described by Sankar *et al*. (2013). The culture supernatant (2 ml) was mixed with hydrocarbons such as diesel, petrol and kerosene and vigorous vortexing was done for 3 min. Later, the tubes were left for 24 h without any disturbance. After 24 h, emulsification activity was estimated as height of emulsified layer/total height x100 (Cooper and Goldenberg, 1987). The experiment was carried out in triplicate and the data presented as mean±SE.

*Bacterial adhesion to hydrocarbons (BATH) assay*

Hydrophobicity of microbial cell surface was measured by BATH assay as described by Rosenberg *et al*. (1980) with slight modification. Cells in the exponential growth phase were harvested by centrifugation at 4000 rpm for 10 min and suspended in phosphate buffered saline to optical density corresponding to 0.5 (AB0). Then diesel was added to the bacterial suspension in the ratio of 3:1 and vortexed for 10 min at high speed and was left undisturbed for 30 min. Then the optical density of the aqueous phase was measured (AB). The degree of cell hydrophobicity was calculated as: AB0-AB/AB0 x 100, where AB0 is the optical density reading before adding

diesel and AB is the optical density reading of aqueous phase (Maneerat and Dikit, 2007). The experiment was carried out in triplicate and the data is given as mean±S.E.

*Oil spreading method*

The overnight grown bacterial strains were centrifuged for 10 min at 4000 rpm and the supernatant was collected. Petri plate was filled with 50 ml of distilled water and 20 µl of diesel oil was added uniformly on the surface of distilled water. Then 10 µl of culture supernatant was poured on the oil surface and observed for the formation of clear zone, which is an indication of biosurfactant production (Satpute *et al*., 2008). The experiment was carried out in triplicate and the data is given as mean±S.E.

*Identification of the bacterial isolate*

Biochemical identification was carried out as per Bergys Manual (Brenner *et al*., 2004). Molecular identification of the bacterial isolate was carried out by 16S r-DNA gene sequencing as per Joseph *et al*. (2015) using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1544R (5'-AGAAAGGAGGTGATCCAGCC-3').

*Optimisation studies*

Various parameters were optimised using liquid BH broth for higher production of biosurfactant by the bacterial isolate BHI 9. The observed parameters included (a) temperature, (b) carbon source (diesel oil was replaced with different carbon sources), (c) Nitrogen source, (d) pH and (e) NaCl (Table 1). Emulsification index and bacterial density ($OD_{600}$) were measured with the parameter under study as variable one and all other parameters as constant. Series of experiments were conducted in triplicate separately for each parameter and the data is given as mean±S.E.

Table 1. Parameters used for biosurfactant production by *Pseudomonas aeruginosa* and their range

| Parameter | Range |
|---|---|
| Temperature (°C) | 25, 30, 35 and 40 |
| Carbon source (1%) | Glucose, Sucrose, Maltose and starch |
| Nitrogen source (1%) | Peptone, Ammonium nitrate, Beef extract and Yeast extract |
| pH | 6, 7, 8, 9 and 10 |
| NaCl (%) | 0.50, 1, 1.50 and 2 |

## Results and discussion

*Isolation and screening of biosurfactant producing bacteria*

A total of 43 distinct morphological isolates from BH agar plates were picked, purified and screened for biosurfactant production using different methods. Fourteen biosurfactant producing bacterial isolates were selected based on the formation of dark blue halos on CTAB agar (Fig. 1), emulsification index (Fig. 2), oil spreading assay and BATH assay. Several methods are available for screening of potential biosurfactant producing microorganisms. Cetyl trimethyl ammonium bromide (CTAB) agar is most commonly used for screening biosurfactant production based on the ability to form clear halo in methylene blue (Lin *et al*., 1998). In this study also the bacterial isolates produced dark blue halo in agar plate.

Among the tested isolates, the bacterial isolate BHI 9 showed highest biosurfactant production in terms of oil spreading assay (3.5 cm), BATH assay (25.3%) and emulsification index on diesel (25%) petrol (31.4%) and kerosene (40%) (Table 2). Emulsification assay is an indirect method used for screening bacterial isolates for biosurfactant production (Thavasi *et al*., 2011). The culture broth may contain the biosurfactant produced

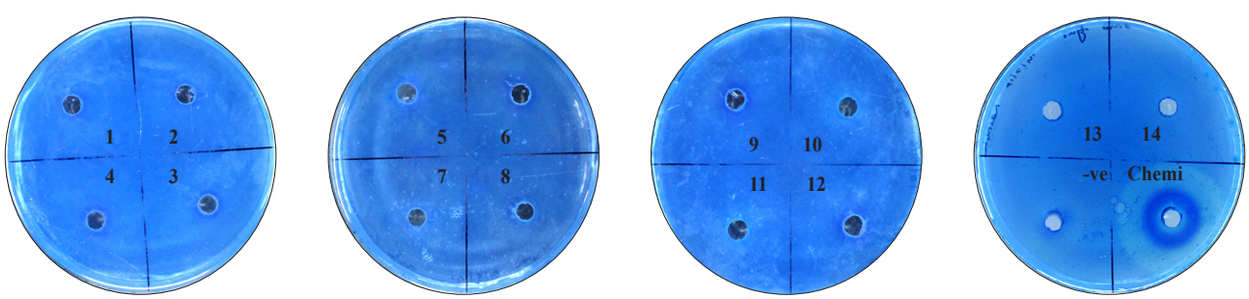


Fig. 1. Bacterial isolates showing biosurfactant production on CTAB agar. Formation of dark blue halo indicates the pairing between anionic biosurfactant and cationic CTAB-methylene blue agar complex. a, b, c, d: Biosurfactant producing bacterial isolates (1-14), -ve : Negative control (distilled water), Chemi: Positive control.(TritonX, chemical detergent)

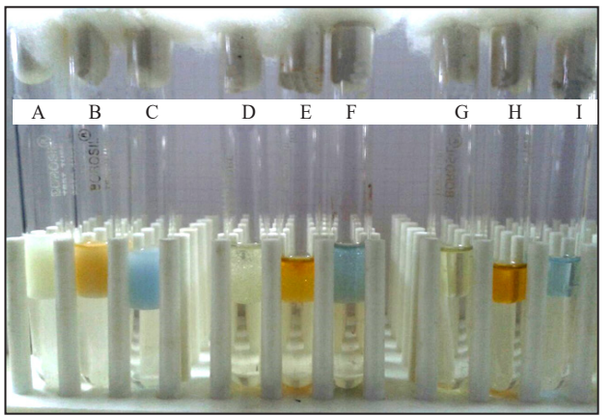


Fig. 2. Emulsification activity of bacterial isolate BHI 9 for biosurfactant production. Test tubes A, D, G - diesel; B, E, H - petrol; C, F, I - kerosene. Triton X (chemical surfactant) is used as positive control (test tubes A, B, C). Bacterial isolate BHI 9 (test tubes D, E, F). Sterile distilled water was used as negative control (test tubes G, H, I)

*Identification of the bacterial isolate*

Biochemical identification of the bacterial isolate BHI 9 revealed that the bacterium is Gram negative, long and slender rod, oxidase positive and catalase positive. Molecular characterisation showed that the bacterial isolates belonged to *P. aeruginosa* (Fig. 3). The sequences were submitted in NCBI GenBank with the accession no. MH050428.

*Optimisation studies*

The biosurfactant production by BHI 9 was optimised under different conditions. Emulsification index and bacterial biomass was found maximum at 35°C, glucose as carbon source, yeast extract as nitrogen source, pH 8 and NaCl 1.5% (Fig. 4 and 5). Growth of the bacterial cells and the biosurfactant production depends on composition of the medium, agitation, temperature and pH (Desai and Banat, 1997; Sahoo *et al*., 2011). In addition, salt concentrations in the medium also show

Table 2. Screening of bacterial isolates for biosurfactant production by emulsification index, oil spreading assay and BATH assay

| Isolate | Emulsification index (%) | | | Oil spreading assay (cm) | BATH assay (%) |
|---|---|---|---|---|---|
| | Diesel | Petrol | Kerosene | | |
| B2F15 | 2.5 | 8.5 | 2.8 | 0.3 | 3.13 |
| B2F14 | 2.5 | 2.8 | 2.8 | 1 | 8.54 |
| B9F12 | 7.5 | 14.2 | 5.71 | 3 | 20.2 |
| B2F9 | 2.5 | 2.8 | 2.8 | 1.8 | 5.3 |
| B2H7 | 2.5 | 2.8 | 2.8 | 0.8 | 3.9 |
| B2F16 | 2.5 | 5.71 | 5.71 | 1 | 10.3 |
| BHA12 | 5 | 5.71 | 5.71 | 0.8 | 5.51 |
| B2H6 | 5 | 5.71 | 5.71 | 2 | 7.43 |
| BHA11 | 2.8 | 2.8 | 2.8 | 1.3 | 4.39 |
| BHA10 | 2.5 | 5.71 | 2.8 | 1.5 | 2.3 |
| B2H8 | 2.5 | 2.8 | 2.8 | 1 | 1.2 |
| BHA9 | 25 | 31.4 | 40 | 3.5 | 25.3 |
| BHA6 | 12.5 | 15 | 10 | 3 | 14.9 |
| BHA8 | 7.5 | 10 | 5 | 2 | 6.4 |

Values are represented as average of triplicate readings

by microorganisms as an excretory product which is responsible for emulsification of hydrocarbon. Oil spreading assay is a rapid and easy method which is used for screening of biosurfactant producing bacteria (Youssef *et al*., 2004). It measures the diameter of the zone formed on the oil surface when biosurfactant is present in the sample. The isolate BHI 9 formed a zone of 3.5 cm on the oil surface. BATH assay is also a simple and indirect method for screening cell hydrophobicity of the biosurfactant producing bacteria. If the bacteria is having the property of adhering to hydrocarbon, that will be useful for determination of degree of bacterial adherence. In this study BHI 9 showed 25.3% bacterial adherence.

effect on biosurfactant production by altering cellular activity ((Ilori *et al*., 2005; Gapke *et al.*, 2007). Water soluble compounds such as glucose and sucrose have been reported as a carbon source for biosurfactant production (Rahman *et al*., 2002). Tuleva *et al*. (2002) reported that *Pseudomonas putida* 21BN strain produced highest emulsifying activity when soluble substrates (glucose) or poorly soluble substrates (hexadecane) were used as carbon source. Similarly, Sahoo *et al*. (2011) observed that *P. aeruginosa* OCD1 produced maximum biosurfactant at 1% inoculum, 2% n-octane as carbon source, optimum temperature between 30 and 35°C, shaking speed 125 rpm and pH 6. Gomathy and Senthilkumar (2013) optimised

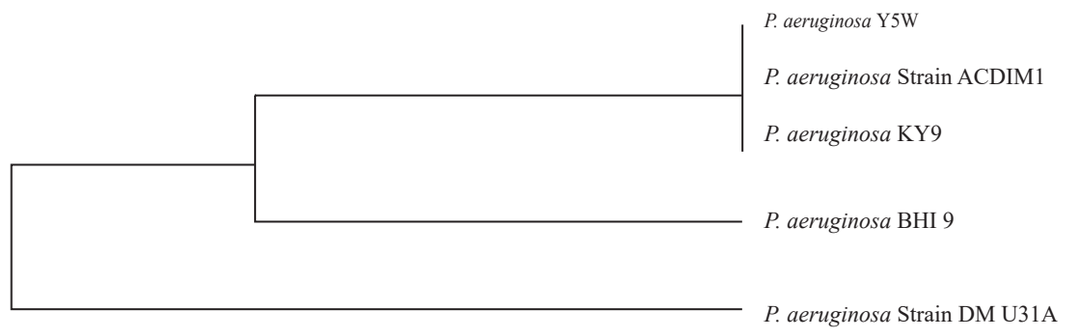


Fig. 3. Phylogenetic tree of the biosurfactant producing bacterial strain *Pseudomonas aeruginosa* BHI 9 based on the 16S rDNA gene sequences (neighbor joining tree method). The reference strains were collected from the gene sequences available in NCBI (BLASTn) domain

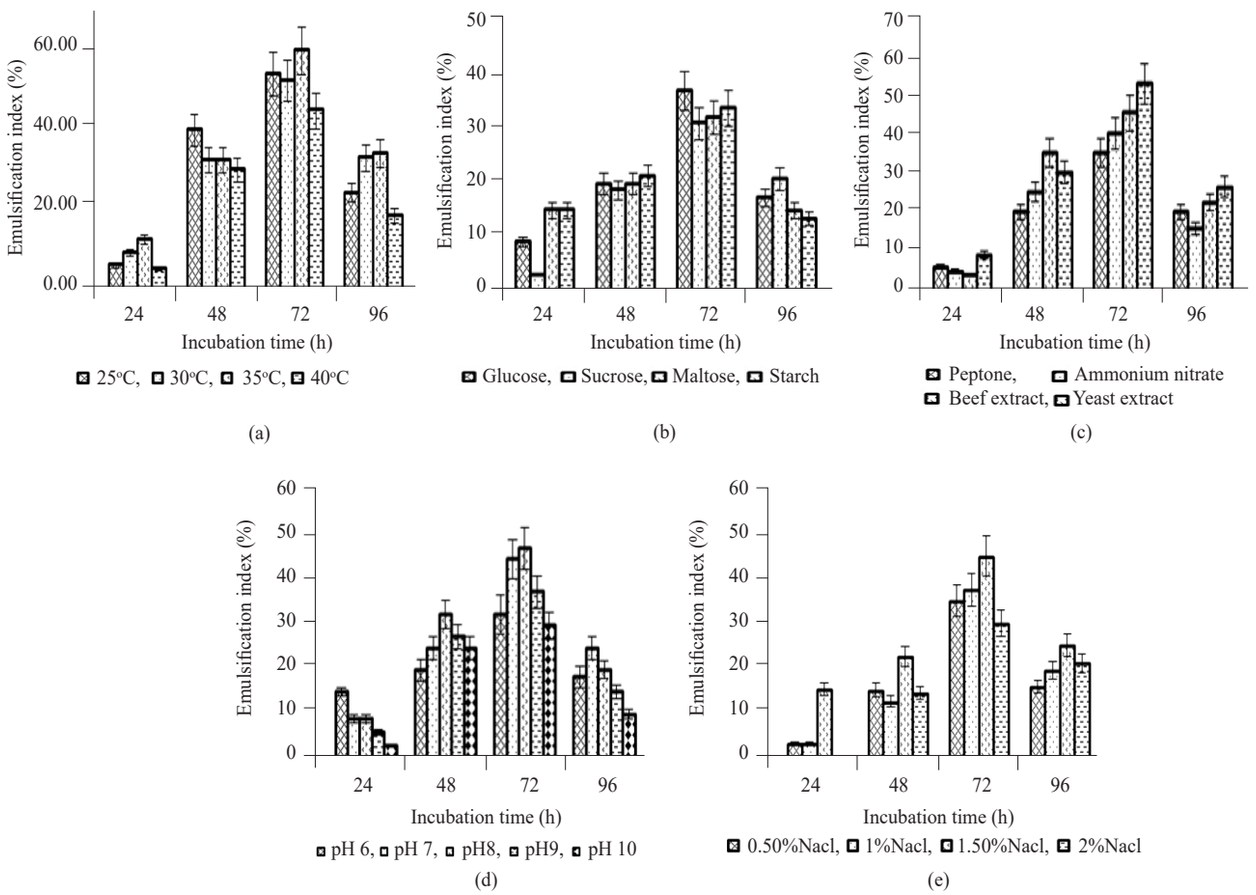


Fig. 4. Emulsification index* for biosurfactant production by *Pseudomonas aeruginosa* BHI 9 at different (a) temperatures; (b) carbon source; (c) nitrogen source; (d) pH and (e) NaCl. Incubation was carried out at 30$^0$C at 125 rpm for 24, 48, 72 and 96 h. *Mean ± S.E, n=3

rhamnolipid production by *P. aeruginosa* at 35°C, pH 9, NaCl 1.5%, ammonium nitrate as nitrogen source and sucrose as carbon source. Sadatfaraji *et al*. (2016) showed biosurfactant production by *P. aeruginosa* was best at 37°C and pH of 6.5-7.5.

In the present study, indigenous biosurfactant producing bacteria isolated and identified from aquaculture farm soil, particularly *P. aeruginosa* (BHI 9) showed good biosurfactant activity. The optimum conditions for maximum biosurfactant production by the

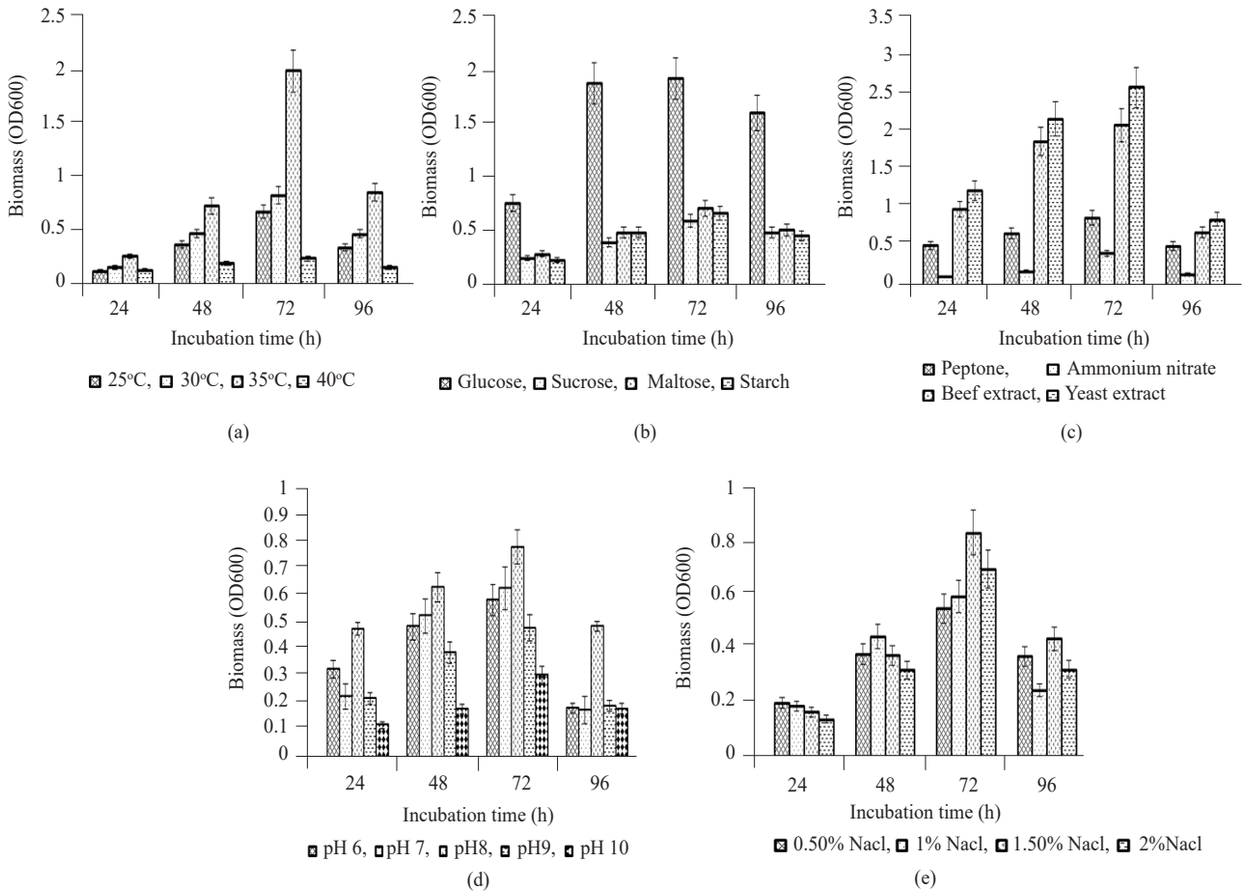


Fig. 5. Bacterial biomass *($OD_{600}$) recorded by *Pseudomonas aeruginosa* BHI 9 at different (a) temperatures; (b) carbon source; (c) nitrogen source; (d) pH and (e) NaCl. Incubation was carried out at 30°C at 125 rpm for 24, 48, 48 and 96 h. *Mean ± S.E, n=3

bacterial isolate BHI 9 was obtained at temperature 35°C, pH 8, NaCl 1.5%, glucose as carbon source and yeast extract as nitrogen source. The biosurfactant produced by *P. aeruginosa* would find applications in bioremediation, food industry, pharmaceutical sector and cosmetics industry. In aquaculture, it could be used as binding agent during feed preparation and as antimicrobial agent.

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