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ORIGINAL ARTICLE **OPEN ACCESS**

Screening and Optimization of Biosurfactant Producing *Pseudomonas aeruginosa*(ATCC 39324)

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ABSTRACT
Surfactant also known as surface-active agents are substances such as detergents that when added to a liquid, reduces their surface tension. Biosurfactants are contrasted groups of surface-active agents produced extracellularly by various bacterial and fungal families. The most effective type of biosurfactant is glycolipid produced by Pseudomonas aeruginosa which is superior to chemical surfactants. Pseudomonas aeruginosa(ATCC 39324) was selected for the production and optimization of biosurfactants. To interpret the effect of the concentration of Sesame seed oil, Engine oil, Soyabean oil, Coconut oil, and Sunflower oil, the study was done to yield a biosurfactant by Pseudomonas aeruginosa. The variation of growth and surfactant development appeared for all the optimization conditions considered. The Pseudomonas aeruginosacultured and produced biosurfactant when grown in different forms of substrates at pH 7 and temperature 25°C. The best substrate for biosurfactant production by Pseudomonas aeruginosa with a great emulsified range is soybean oil. The biosurfactant induced by Pseudomonas aeruginosa emulsified different carbon sources and nitrogen sources. This study further showed that Pseudomonas aeruginosa has a greater capability of bioremediation in the case of oil-contaminated water resources.
Keywords: *biosurfactant,bioremediation,emulsification, optimization.*
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INTRODUCTION

Biosurfactant is different in nature fromsurface-active emulsifier compounds synthesized by various types of microorganisms. Their exclusive properties, such as high point biodegradability, little toxicity, environmental potentiality, and gentle production condition have made them aware of their excellent applications in several industries. The chemical structure of biosurfactantsis distinct from others with varieties in carbon sources, microorganisms, and other molecular structures[1].

The types of biosurfactants are lipopeptides produced by *various Bacilli,* glycolipids produced by *Pseudomonas* &*Candida sp.,* phospholipids produced by *Thiobacillus sp.,*polysaccharide- lipid compounds produced by *Acinetobacter sp.* or even microbial cell surface acting itself as biosurfactant. Biosurfactants have both polar & non-polar domains. So, they are able to separate at water-oil surfaces which reduces surface tension. These surface characteristics made them a good option for the recovery of oil known as enhanced oil recovery (EOR)[2]. Lipopeptides and glycolipids are present in less molecular weight biosurfactantsas well as proteins and polysaccharides present in high molecular weight biosurfactants[3]. Glycolipids and phospholipids are generally the most studied and foremost isolated group of biosurfactants. Biosurfactants are beneficial as they provide low irritancy and low affinity with human skin[4].Carbohydrates, hydrocarbons, or aggregation of both are necessary substrates for the manufacturing of biosurfactants. Surface assimilation is the mechanism of action involved in biosurfactant where the tension between hydrocarbon, and microbial phases arenecessarily decreased and the phases obtain a greater tendency to combine with each other [5].

The broad applications o fbiosurfactants are in oil restoration, microbial bioprocesses, and cosmetics. To control the virulence of synthetic compounds, the use of biosurfactants may considera good substitute[6]. Biosurfactant synthesizing microorganisms has a future application for correcting a situation of hydrocarbon-contaminated sites. Biosurfactants are also useful in various industrial applications such as the pulp industry, pharmaceutical industry, agricultural industry, food industry, and petroleum industry[7].Biosurfactant has a role in lipid digestion and has ecological uses mainly related to the biodegradation of petroleum hydrocarbons in water found in underground soil and the humiliation of harmful compounds. In the oil industry, biosurfactants are used in the washing of uncleaned vessels and to guide the shipping of heavy crude oil in pipelines [8]. Biosurfactants enhance the usability of non-soluble carbon sources incorporated in the media to the cells [9].

The present study was conducted on the screening and optimization of biosurfactant-producing *Pseudomonas aeruginosa*(ATCC 39324). The optimization was carried out using different environmental factors like carbon source, nitrogen source, pH, temperature, and substrates[10].

MATERIALS AND METHODS

Screening of *Pseudomonas aeruginosa*(ATCC 39324)for biosurfactant production

A Bushnell Hass Broth (BHB) containing (g/L) composition of 0.2 gm magnesium sulfate ($MgSO_4$), 0.02gm calcium chloride ($CaCl_2$), 1 gm potassium dihydrogen phosphate (KH_2PO_4), 1 gm dipotassium hydrogen phosphate (K_2HPO_4), 1 gm ammonium nitrate (NH_4NO_3), 0.05 gm ferric chloride ($FeCl_3$) was prepared as a selective media for biosurfactant production. 250 ml conical flask was filled with 100ml of BHB media and a loop full of pure culture of *Pseudomonas aeruginosa*was added to the solution. The broth was incubated at a shaking incubator (100rpm) at room temperature (30℃) for 7days[9]. After 7 days of incubation, the culture broth was centrifuged at 10,000 rpm for 15 min. The supernatant was taken for further the assay of oil spreading technique [3].

Oil displacement method

Morikawa et *al* developed the oil displacement method[11]. Distilled water was added to the Petri dish with the addition of engine oil on the surface of the water. Then 120 µL supernatant of enriched centrifuged culture was dropped on oil surface and allowed to stand for 60 sec without disturbing the drop. The zone of clearance on the oil surface was observed and measured the diameter of theclear zone[7].

Emulsification index (E24)

To determine the emulsification index (E24) of samples, 2ml of each oil i.e.,coconuts oil, engine oil, petrol, sunflower oil, soybean oil, and sesame oil added to the 2ml of culture supernatant in the test tube and mixed for 2 min with the help of vortex mixture at high speed and leave to stand undisturbed for 24 hours [7]. The height of the stable emulsified layer is measured with help of a regular scale[11]. The E24 index is given as a percentage of the height of emulsified layer (mm) divided by the total height of the liquid column (mm)[7].

$$E24 = \frac{\text{Height of emulsified layer}}{\text{Height of total solution}} \times 100$$

Blood HemolysisTest

Enriched BHB broth containing *Pseudomonas aeruginosa* was streaked on blood agar (nutrient agar containing 5% blood) plates to detect the hemolysis activity. These plates were incubated at30℃ for 48 hours [8].

Optimization of growth conditions

1.Incubation period: The pure culture of *Pseudomonas aeruginosa* was inoculated in BHB media and incubated in a shaking incubator at 30℃. Afterincubation of 24, 48, 72, 96, 120, 144, and 168 hours, the enriched broth was centrifuged at 10,000 rpm for 15 min. By applying oil displacement method, the diameter of the clear zone on the oil surface was observed and recorded every 24 hours. The maximum growth and highest zone were recorded on day 6.This incubation period was used for other optimization conditions.

2.Effect of pH

To check the effect of pH, 5 flasks of different pH ranging from 5-9 of 50 ml BHB media are inoculated with5% of enriched culture of BHB containing *Pseudomonasaeruginosa* and incubated at room temperature for 6 days[12].

3.Effect of temperature

A set of different temperatures was considered to find out the optimum temperature for highest biosurfactant production. Different temperatures as 25, 30, 35&40℃ was examined after addition of 5% enriched culture in each flask containing 50 ml BHB[4].

4. Effect of carbon source

Various carbon sources were optimized to choose one most acceptable for thehighest production of biosurfactant by *Pseudomonas aeruginosa*. The carbon sources used in this study were glucose, fructose, xylose, maltose. They were inoculated with 5% enriched culture in 50 ml BHB media flasks and incubated at room temperature for 6 days[4].

5.The effect of nitrogen source

To check the effect of nitrogen sources such as potassium nitrate (KNO_3), ammonium chloride (NH_4Cl), sodium nitrate ($NaNO_3$), ammonium nitrate (NH_4NO_3) was examined for highest production of biosurfactant. For this step, the nitrogen sources were used at 1% concentration and inoculated along with 5% enriched culture in each flask of 50 ml BHB media[4].

6. The effect of substrate

The substrates used in this optimization were Coconut oil, Sesame oil, Soyabean oil,and Sunflower oil. They were added as 1% in aflask containing 50 ml BHB media along with 5% enriched culture. Flasks were incubated in a shaking incubator at 30°C and 100 rpm for 6 days[1].

E24 index for each optimized flask

E24 was determined for each flask by mixing 2 ml ofbiosurfactant with an equal volume of substrate used i.e., Soybean oil in a test tube and mixed by using a vortex mixer for 2 min. Tubes were left to stand for 24 hours. The E24 is indicated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm)[4]. E24 was recorded after 24 hours of incubation.

RESULTS

Oil displacement method:

Oil displacement method was performed to determine the ability of biosurfactant to form clear zone on a plate containing oil and water. The enriched culture supernatant shows the zone of clearance on the plate (fig.1).

Fig. 1. Oil displacement method

Emulsification index (E24):

The emulsification index test was used to check whether the biosurfactant has emulsifier properties or not. The emulsification index was calculated (table 1) according to the formula for each sample. After 24 hours of incubation, Soybean oil shows a maximum E24 index while petrol shows the lowest E24 index.

Table 1: Emulsification Index (E24) in percentage

Sample	Height of emulsified layer in mm	Height of total solution in mm	Emulsification index (E24)
Coconut oil	17	27	62.96 %
Sunflower oil	12	27	44.44 %
Soybean oil	19	28	67.85 %
Sesame oil	15	27	55.55 %
Petrol	8	23	34.78 %
Engine oil	13	27	48.14 %

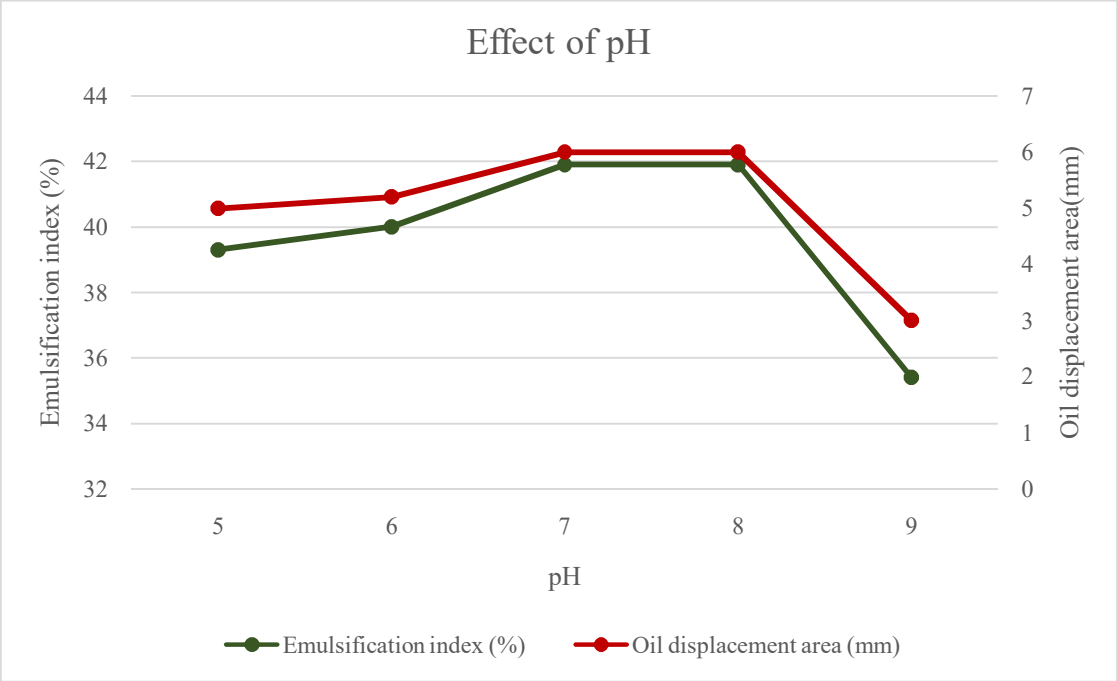
Blood hemolysis test:

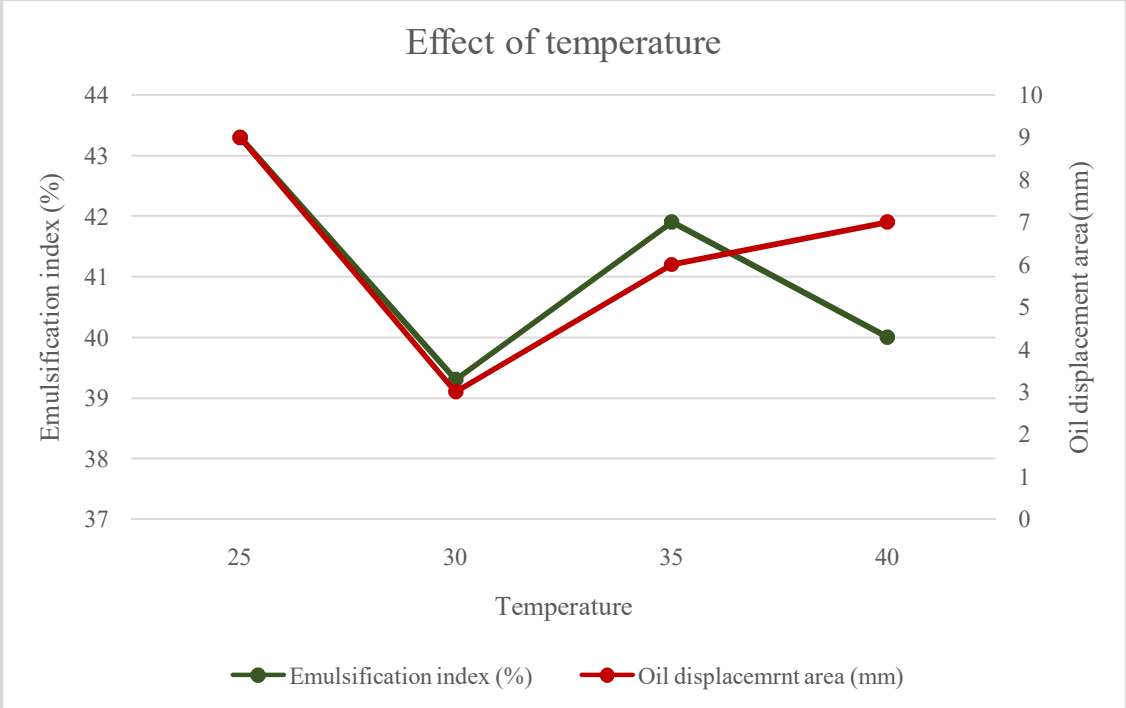
Enriched loopful culture streaked on the blood agar plate shows the zone of hemolysis (Fig.2). *Staphylococcus aureus* shows alpha hemolysis on the blood agar plate.

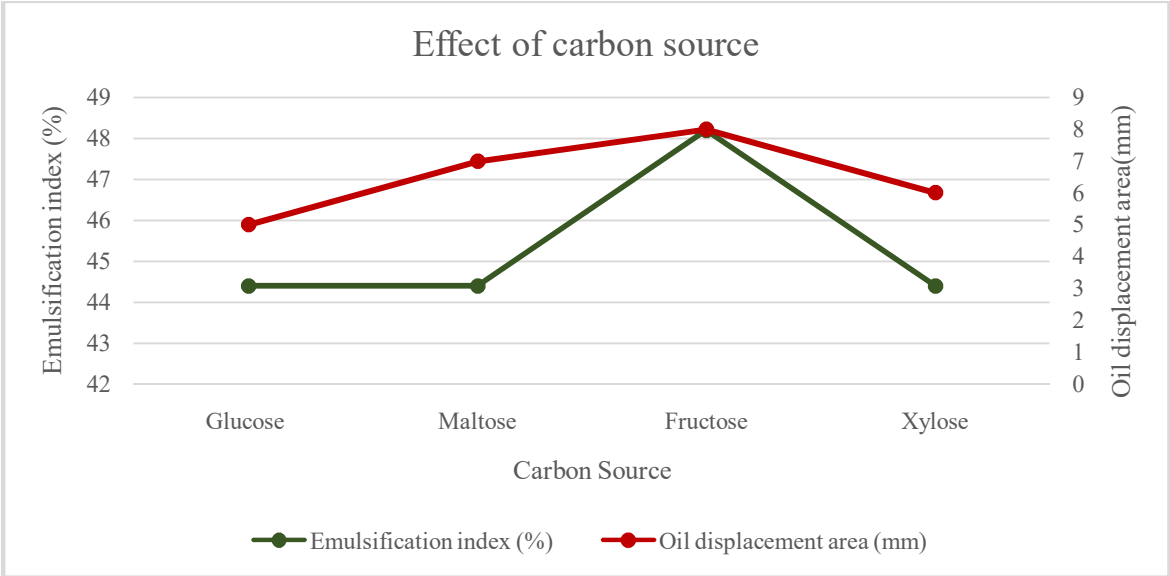
Fig. 2. Blood agar plate showing zone of hemolysis around the colonies

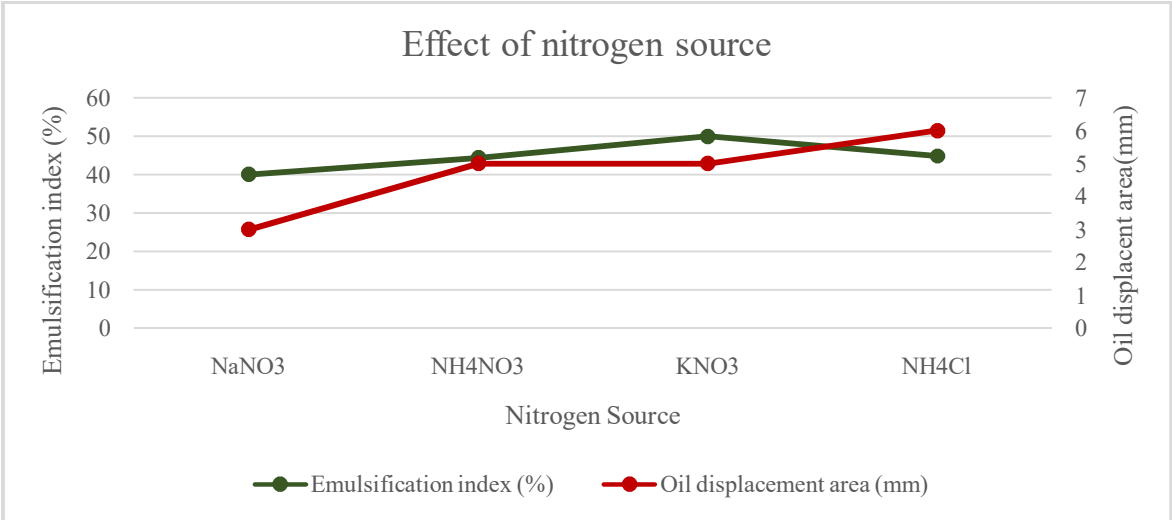
Optimization of growth condition

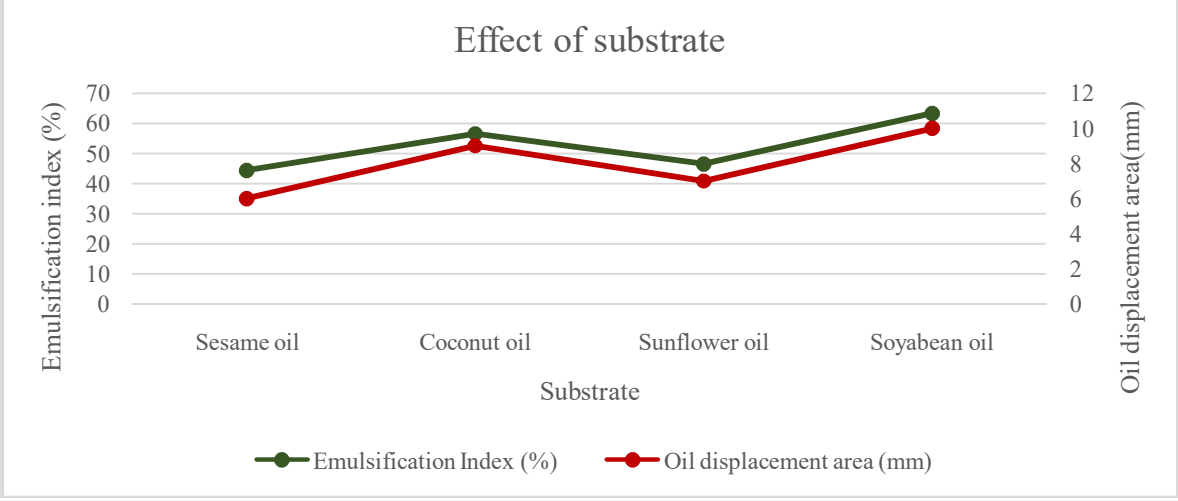
The maximum zone was observed on the 6th day of the incubation period. After that, optimization for different parameters such as pH, temperature, carbon source, nitrogen source, and substrate were carried out by using the oil displacement method and emulsification index. Comparative analysis was studied for each optimized condition by measuring the oil displacement area and emulsification index.






Effect of carbon source
Emulsification index (%)
Oil displacement area(mm)
49
48
47
46
45
44
43
42
9
8
7
6
5
4
3
2
1
0
Glucose
Maltose
Fructose
Xylose
Carbon Source
Emulsification index (%)
Oil displacement area (mm)


Effect of nitrogen source
Emulsification index (%)
Oil displacent area(mm)
60
50
40
30
20
10
0
7
6
5
4
3
2
1
0
NaNO3
NH4NO3
KNO3
NH4Cl
Nitrogen Source
Emulsification index (%)
Oil displacement area (mm)


Effect of substrate
Emulsification index (%)
Oil displacement area(mm)
70
60
50
40
30
20
10
0
12
10
8
6
4
2
0
Sesame oil
Coconut oil
Sunflower oil
Soyabean oil
Substrate
Emulsification Index (%)
Oil displacement area (mm)

DISCUSSION

The screening and optimization of biosurfactant-producing *Pseudomonas aeruginosa* (ATCC 39324) are determined by using the emulsification index (E24) and Oil displacement method [1]. The diameter of the clear zone observed by the oil displacement method was up to9 mm. The superior emulsification index performed with biosurfactant produced by *Pseudomonas aeruginosa* was 48.14% for Soyabean oil (Table 1). Then further screening of *P.aeruginosa*for biosurfactant production was done by hemolytic activity. It was found that the clear zone formed around the colonies on blood agar plates [6]. The choice of a proper pH, temperature, carbon source, nitrogen source, and substrate are one of the most crucial steps in the improvement of the systematic and cost-effective biosurfactant production process [10]. Biosurfactant production gained its maximum value at 30°C, with an optimal pH range of 7-8 while low at pH value 9. In this recent work, the optimum temperature for biosurfactant production was found at 25°C and the lowest production was found at 30°C. Fructose was found to be the leading carbon source with 8 mm of the clear zone of diameter and the poor carbon source was glucose with a 5 mm diameter. Greatest bacterial growth was observed in Potassium nitrate (KNO_3) containing BHB medium, with almost equal growth being recorded for the rest of the two nitrogen sources (Ammonium chloride and Ammonium nitrate). The lowest bacterial growth was observed in Sodium nitrate ($NaNO_3$). In the case of substrates, Soybean oil enhances biosurfactant production with a diameter of the clear zone up to 10 mm while in the case of Sesame oil the diameter was 6 mm (Table 2).

The E24 value was maximum for pH 7 and 8 (41.9%) and minimal for pH 9 (35.4%). A maximum value of E24 was observed for temperature 25°C (43.3%) and a minimum for 30°C (39.3%). In the case of carbon source, xylose, glucose, and maltose are almost the same had 44.4 %ofE24meanwhile for fructose, the E24 value observed is 48.2%.The E24 reached a maximum percentage of up to 50% for Potassium nitrate (KNO_3) and a minimum of 40% for Sodium nitrate ($NaNO_3$). The highest E24 index was found at 63.3% for Soybean oil and the lowest E24 index was found at 44.4% for Sesame oil (Table 3). These results indicated that a large amount of biosurfactant production was as per suitable conditions of optimization factors such as pH, temperature, carbon source, nitrogen source, and substrates used.

CONCLUSION

Pseudomonas aeruginosa (ATCC 39324) is a very implicit biosurfactant-producing indigenous strain. *Pseudomonas spp.* are known for their wide nutritional adaptability which allows them to use many debris and contaminants as a source of carbon & energy. Biosurfactant production was efficiently optimized using various methodologies. From this study, we suggested that *Pseudomonas aeruginosa* (ATCC 39324) is suitable as well as the best candidate for biosurfactant production and bioremediation.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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