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RESEARCH ARTICLE

Optimization of culture conditions for biosurfactant production by *Wickerhamomyces edaphicus* isolated from Mangrove region of Mundra, Kutch, Gujarat

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Abstract

Objectives: Biosurfactants are surface active compounds capable of reducing surface tension and interfacial tension. The selection of low cost and renewable sources are essential for successful biosurfactant production. The aim of this study is to optimize the media and culture conditions for the biosurfactant production by *Wickerhamomyces edaphicus* using cottonseed oil as a single Carbon source. **Methodology:** *W. edaphicus* was isolated from the mangrove areas of Mundra, Gujarat, and screened for the production of biosurfactant by oil displacement test and emulsification index method. The production media were optimized for maximum biosurfactant production from *W. edaphicus* by optimizing the cultivation conditions such as carbon source, nitrogen source, temperature, pH and inoculum size. **Findings:** *W. edaphicus* could be a potential yeast for biosurfactant production using cottonseed oil as a carbon source. During the optimization process, the optimal growth parameters obtained were: cottonseed oil 4% (emulsification index: 66.8%) and yeast extract 0.1% (emulsification index: 63.33%) and the environment parameter were: temperature 30°C (emulsification index: 64.51%) and pH 6.0 (emulsification index: 66.54%). **Novelty/Applications:** Biosurfactant produced by *W. edaphicus* finds wide variety of applications in detergent, food , biotechnological, pharmaceutical and cosmetic industries, oil recover enhancement and bioremediation.

Keywords: Biosurfactant; cottonseed oil; emulsification index; oil displacement; Wickerhamomyces edaphicus

1 Introduction

Biosurfactants are amphipathic molecules having both hydrophilic and hydrophobic moieties. Biosurfactants have the ability to reduce surface and interfacial tensions by accumulating at the interface between two immiscible fluid phases[(1)]. Biosurfactants are surfactants produced by microorganisms, mostly in microbial cell surfaces or excreted extracellularly[(2)]. Comparing with chemical surfactants, biosurfactants have several advantages such as lower toxicity, higher biodegradability, higher foaming capacity and higher activity at extreme temperatures, pH levels and salinity[(3–5)]. Biosurfactants have applications in variety of industries like pharmaceuticals, cosmetics, food, crude oil recovery, as antimicrobial agents in health care, detergent and agricultural industries[(4,5)].

Due to high production costs, biosurfactants are not able to compete economically with chemical surfactant. High production cost of biosurfactants results from the use of expensive substrate. Therefore, the selection of cheap and renewable source is essential[(6)]. The best solution for renewable source would be the re-utilization of industrial wastes, for instance, the agro-industrial or the oil-containing wastes[(7)]. The oils are attractive substrate for the biosurfactant production. Many food industries and oil refinery industries produced large quantities of wastes. Renewable sources from different varieties of waste oils like, soybean oil, olive oil, corn oil[(8)], sunflower oil[(9)], peanut oil[(10)], canola oil[(11)], cotton seed oil[(8,12)] used to minimize the cost of substrate in biosurfactant production. In this study, cottonseed oil has been used as substrate for production of biosurfactant.

Optimization of the growth media and cultivation conditions are important in order to get maximum biosurfactant production[(6)]. Production of biosurfactant and growth of organism are strongly affected by medium composition: carbon sources, nitrogen sources, growth factors and Environmental factors such as pH, temperature, inoculum age, inoculum size, agitation, fermentation period through their effects on cellular growth or activity. The classical method of medium optimization involves changing one variable at a time, keeping the others at fixed levels[(13)].

In this study, production of biosurfactant from the yeast *Wickerhamomyces edaphicus* using cottonseed oil as a low cost carbon substrate and optimization of the growth media and environmental factors for maximum biosurfactant production was studied.

2 Materials and Methods

2.1 The sampling sites

In this study, the soil samples were collected from the mangrove areas of Mundra, Kutch region of Gujarat in India (Latitude – 22°46'230"and Longitude – 69°42'141"). Samples were collected from rhizospheric areas of mangroves and stored in sterile plastic bags at 4°C.

2.2 Isolation of mangrove yeast

Mangrove yeasts were isolated by using Yeast Malt Extract Broth (YM) with composition; 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, pH 7 and supplemented with 0.025% sodium propionate and 200 mg chloramphenicol[(14)]. 0.1g of soil samples were inoculated into 100ml flask containing 25 ml YM broth. Flasks were incubated at 120 rpm and 30°C for 72 h. After incubation period, broth was serially diluted (10° to 10^{-6}). 100 μl of broth from each dilution was inoculated on the YM plates. Yeast colonies were purified and selected based on their morphology.

2.3 Screening of biosurfactant producing yeasts

Minimal Salt Medium (MSM) as described by Kitamoto et al. 1990 (0.03% KH_2PO_4, 0.03% $MgSO_4$, 0.3% $NaNO_3$, 0.1 Yeast extract, 4% Glucose, pH 7) was used for biosurfactant production with slight modification in use of substrate[(15)]. 4% cottonseed oil as a carbon substrate in place of 4% glucose was incubated at 30°C in shaking condition at 120 rpm for 10 days. After incubation, the media were centrifuged at 10,000 x g for 15 min. The supernatants were collected and biosurfactant production was examined with oil displacement test and emulsification activity.

2.3.1 Oil displacement test

100 μl of crude oil was placed onto the surface of 40ml of distilled water in a petriplate. 10 μl of the culture supernatant obtained earlier was added into the centre of petridish and observed for a clear zone. Oil displacement was measured as the activity of surfactant[(16)].

2.3.2 Emulsification measurement

4 ml of culture supernatant and 4 ml of kerosene was added in test tube and vortexed at high speed for 2 min. The mixture was allowed to stand for 24 h. The emulsification activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage[17].

$$Emulsification\ activity\ (E\ 24)\ = \frac{Height\ of\ emulsion\ layer}{Total\ height} X\ 100 \quad (1)$$

2.4 Optimization of biosurfactant production

Minimal Salt Medium was used for optimization of biosurfactant production. All the experiments were performed in triplicate.

2.4.1 Effect of different oils as a carbon source on the production of biosurfactant

100ml of MSM medium with concentration of different substrate olive oil, soybean oil, paraffin oil and cottonseed oil was prepared in different 500 ml Erlenmeyer flasks and incubated at 30°C for 10 days at 120 rpm. The effect of these oils on the production of biosurfactant was evaluated by using emulsification index method.

2.4.2 Effect of varying concentration of selected carbon source on the production of biosurfactant

The MSM medium supplemented with different concentrations (0%, 2%, 4%, 6% and 8%) of cotton seed oil as a carbon source was prepared in 500 ml Erlenmeyer flasks and incubated at 30°C for 10 days at 120 rpm. The effect of different concentration of oil on the production of biosurfactant was determined by emulsification index method.

2.4.3 Effect of nitrogen source on the production of biosurfactant

In 500ml Erlenmeyer flasks containing 100ml of MSM medium with concentration of different nitrogen sources namely ammonium chloride, yeast extract, ammonium nitrate, sodium nitrate and peptone were added. Flasks were incubated at 30°C for 10 days at 120 rpm. Emulsification index was determined for production of biosurfactant.

2.4.4 Effect of temperature on the production of biosurfactant

The MSM medium were prepared into five different flasks containing 100 ml of MSM medium. Flasks were incubated at different temperatures (25°C, 30°C, 35°C, 45°C and 50°C) for 10 days at 120 rpm. The effect of incubation temperature on the production of biosurfactant was measured by emulsification index method.

2.4.5 Effect of pH on the production of biosurfactant

The MSM medium were prepared with five different pH (2, 3, 4, 5, 6, 7 and 8). All flasks were incubated at 30°C for 10 days at 120 rpm. pH was adjusted by using 0.1 N NaOH and 0.1 N HCl. Emulsification assay was determined.

2.4.6 Effect of inoculum size on the production of biosurfactant

Different 500 ml Erlenmeyer flasks containing 100 ml of MSM medium were inoculated separately with different inoculum sizes such as 2.5%, 5.0 %, 7.5% and 10 % (v/v). All the experimental flasks were kept in incubator shaker at 120 rpm for 10 days at 30°C. Emulsification activity was measured.

3 Results and Discussion

3.1 Isolation and screening of biosurfactant producing yeast

From Mundra soil samples, total 24 morphologically different yeast colonies were isolated. Among 24 yeasts, 10 were considered as potential biosurfactant producers. Ky-09 was screened as potential biosurfactant producing yeast based on screening test results including oil displacement and emulsification activity.

3.1.1 Oil displacement method

The clear zone formation on crude oil by supernatant of Ky-09 shows the biosurfactant production. 4 cm clear zone was formed by Ky-09.

3.1.2 Emulsification index test (E_{24})

Emulsion layer was seen in the test tube between the two liquid phases kerosene and culture supernatant of Ky-09 yeast strain. The formation of emulsion layer indicated the production of biosurfactant. The emulsification Index was 62.96 %.

3.2 Identification of biosurfactant producing yeast

The highest biosurfactant producing isolate named Ky-09 was identified as *Wickerhamomyces edaphicus* through 18s RNA sequence analysis at Gujarat Biotechnology Research Centre, Gandhinagar, Gujarat, India. Wickerhamomyces species have ability to grow at 37°C and 40°C, which helps in biosurfactant production at high temperature[18].

3.3 Optimization of various parameters for biosurfactant production

3.3.1 Effect of different substrate on the production of biosurfactant

The isolate *W. edaphicus* was able to utilize cottonseed oil as a sole carbon source and produced higher amount of emulsification index (E_{24}) 67.4 % followed by 46.2% emulsification index by paraffin oil (Figure 1). The soybean oil also utilized by the isolate and shows 32.25% emulsification index. Lowest emulsification index was observed with the olive oil 13.33%. Large amount of foam was observed in the culture medium containing cottonseed oil. The carbon source was found to affect the biosurfactant production to a great extent because biosurfactant is cell-wall associated[19]. The *Candida lipolytica* has showed optimum production in medium containing 6% soybean oil refinery residue and 1% glutamic acid[20]. *Candida antarctica* produced biosurfactant using oleic acid as a substrate[21]. Thavasi et al. used a mixture of peanut oil cake and waste motor lubricant oil as a substrate for the biosurfactant production[10]. *Torulopsis bombicola* produced biosurfactant by using corn oil, soybean oil, sunflower oil and safflower oil as substrate.[22].

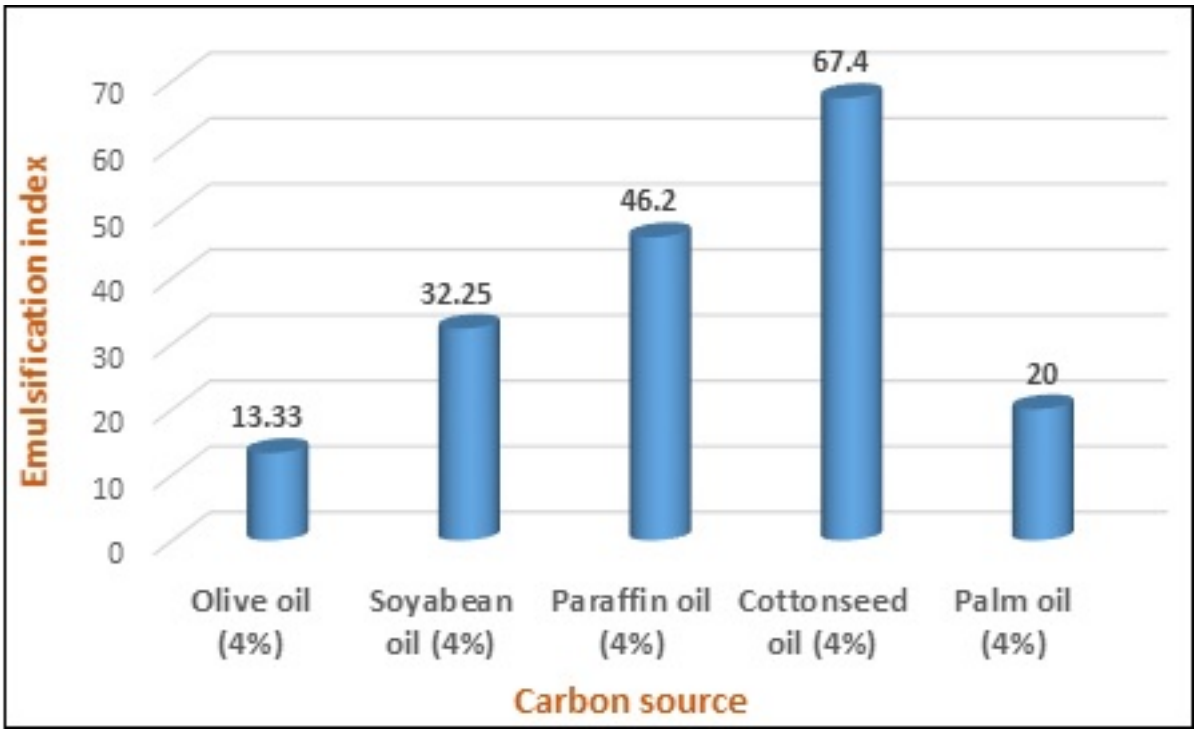

Fig 1. Effect of different oilsas a carbon source on biosurfactant production by *Wickerhamomyces edaphicus*

3.3.2 Effect of different concentration of substrate on the production of biosurfactant

The effect of different concentration of cotton seed oil on the production of biosurfactant was analysed. The results showed that 4% cotton seed oil gives maximum emulsification index 66.8% followed by 46.66 % emulsification index by 2% cotton seed oil. The results are shown in Figure 2. Candida glabrata strain isolated from mangrove sediments produced maximum biosurfactant 10 g/l by using cotton seed oil (7.5%) and glucose (5.0%) as a substrate[23].

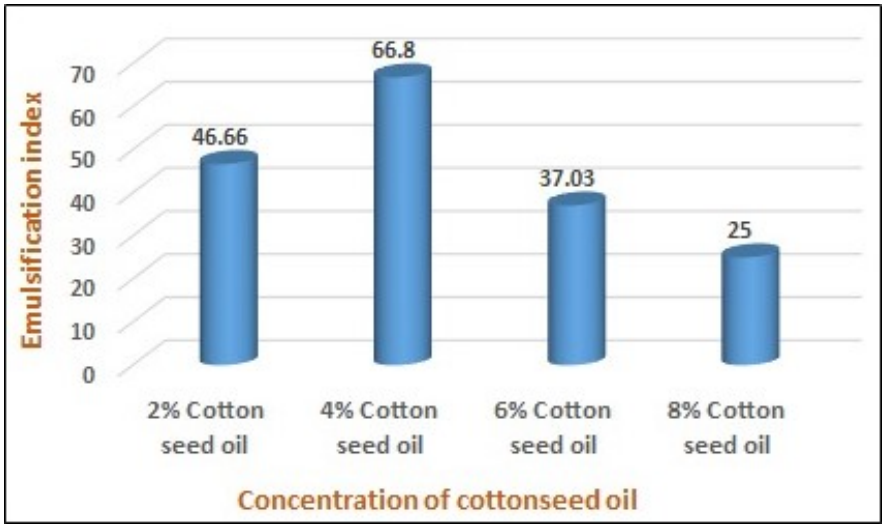

Fig 2. Effect of varying concentrations of cottonseed oil on biosurfactant production by *Wickerhamomyces edaphicus*

3.3.3 Effect of nitrogen source on the production of biosurfactant

Yeast extract was the best source of nitrogen for growth and biosurfactant production by *W. edaphicus* (Figure 3). Emulsification index for yeast extract recorded was 63.33%. Ammonium chloride, and ammonium nitrate, sodium nitrate and peptone showed emulsification index 39.58%, 46.15%, 29.62% and 41% respectively. The nitrogen source in the medium influence the production of biosurfactant[24]. Biosurfactant production was in maximum under limited nitrogen supply[25–27]. Cooper and Paddok observed that 5 g/l of yeast extract produced higher biosurfactant[22]. Casas and Ochoa studied effect of different yeast extract concentration (1 to 20 g/l) on biosurfactant production by *C. bombicola*. According to their study the production of biosurfactant is maximum in the presence of yeast extract concentration 1 g/l. In this study *W. edaphicus* also produced maximum biosurfactant by using 1g/l yeast extract[28].

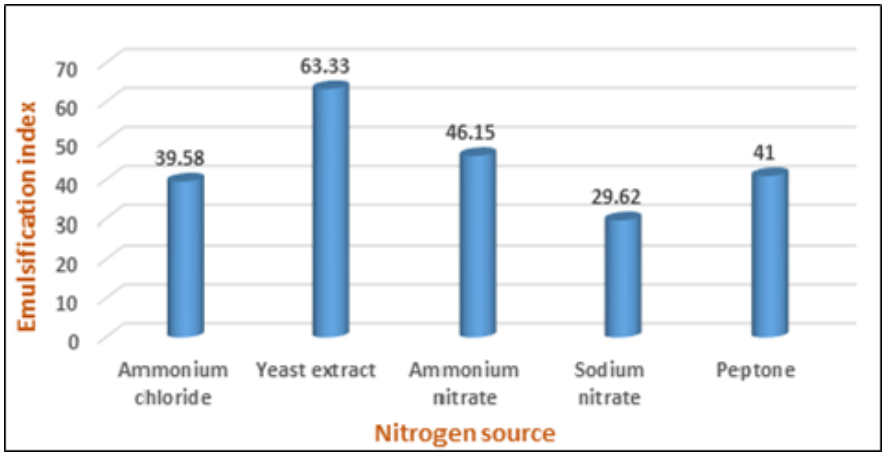

Fig 3. Effect of nitrogensource on biosurfactant production by *Wickerhamomyces edaphicus*

3.3.4 Effect of Temperature on the production of biosurfactant

The effect of varying temperature like 25°C, 30°C, 35°C, 45°C and 50°C was studied on biosurfactant production by *W. edaphicus*. The amount of biosurfactant production was estimated by emulsification index method. The highest biosurfactant production with emulsification index 64.51 %was achieved at temperature 30 °C and followed by 25 °C (emulsification index 45.16%). As shown in Figure 4, after 30 °C, the biosurfactant production decreased. The studies done by various researches prove that optimum biosurfactant production was achieved in the temperature interval of 28-30 °C[27,29–31]. For *Candida tropicalis* opti-

mum production of sophorolipid was at 30°C[32].

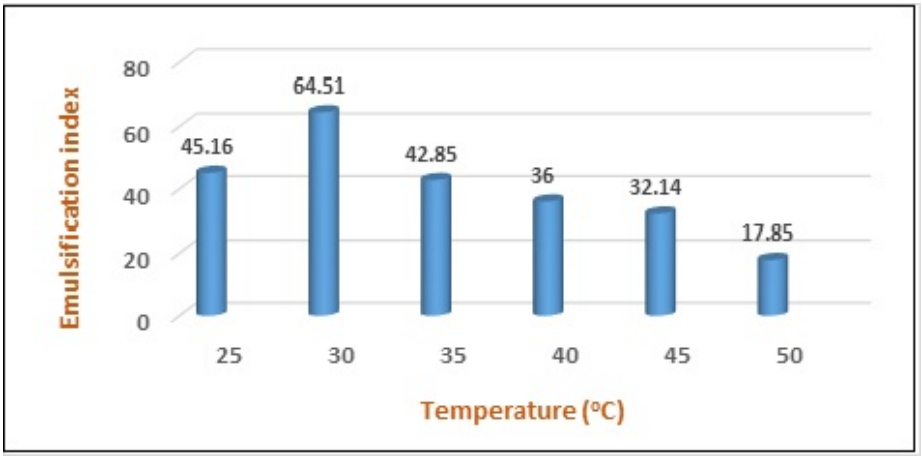

Fig 4. Effect of temperature on biosurfactant production by *Wickerhamomyces edaphicus*

3.3.5 Effect of pH on the production of biosurfactant

The effect of varying pH like 2, 3, 4, 5, 6, 7, and 8 of MSM medium supplemented with 4% Crude cotton seed oil was studied on biosurfactant production. At pH value 6.0 the highest production of biosurfactant was observed with 66.54% emulsification index. Biosurfactant production was increased by increasing medium pH from 3.0 to 6.0. After pH 6.0, a decline in biosurfactant production was observed (Figure 5). Candida tropicalis produced maximum sophorolipid at pH 3.5[32]. Zinjarde and Pant observed the maximum biosurfactant production at pH 8.0[33]. Daverey and Pakshirajan, obtained maximum biosurfactant production at media pH 6.0 using glucose and rapeseed oil[30,31].

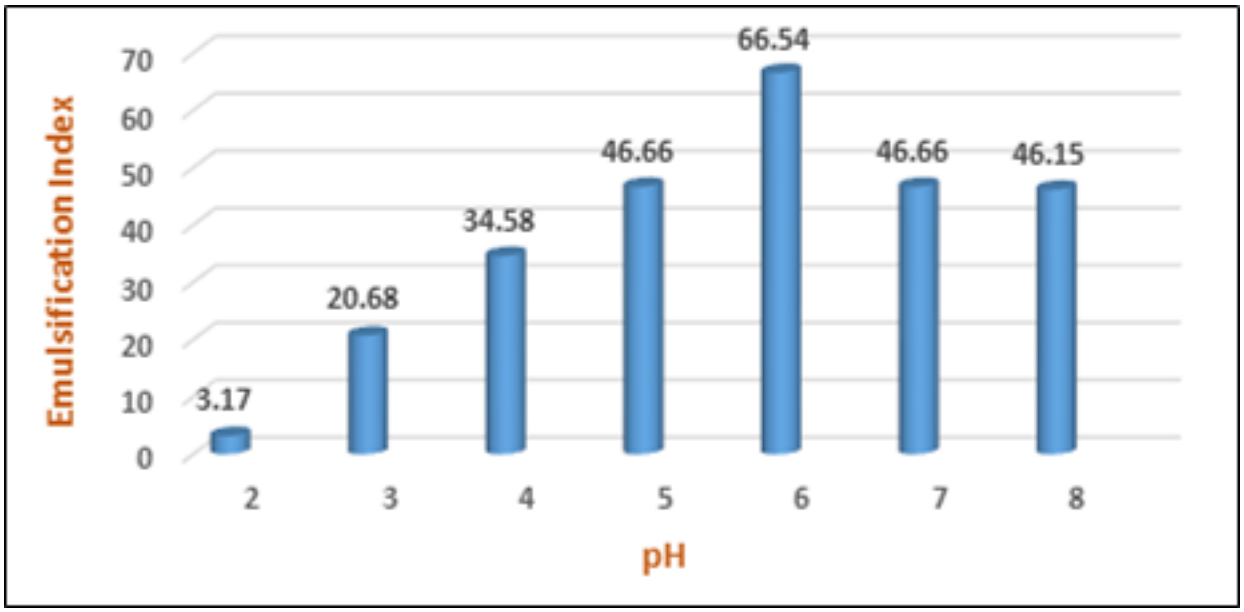

Fig 5. Effect of pH on biosurfactant production by *Wickerhamomyces edaphicus*

3.3.6 Effect of inoculum size on the production of biosurfactant

Varying percentage like 2.5, 5, 7.5 and 10% of inoculum volume were used for the study. As shown in Figure 6, the optimal inoculum size for maximum biosurfactant production with emulsification index 62.96% was observed at 5% inoculum size and

followed by 7.5% (emulsification index 51.72%). Inoculum size is also important for the biosurfactant production. According to Cavalero and Cooper, biosurfactant production was shown maximum at 10% inoculum size[34].

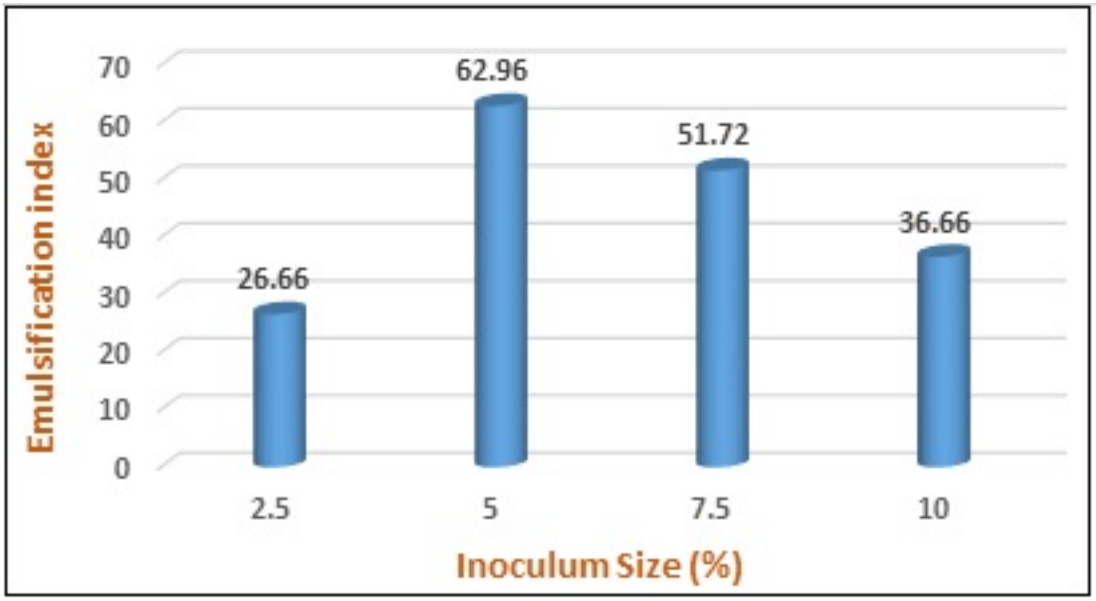

Fig 6. Effect of inoculumsize on biosurfactant production by *Wickerhamomyces edaphicus*

Inoculum age is not much affected on biosurfactant production. It is proven in earlier studies where some authors used 48 h of inoculum age for biosurfactant production[27,28,31] while some authors used 72 h of inoculum age. In this study, 72h of inoculum age was used for biosurfactant production by *W. edaphicus*.

Table 1. Optimum levels for biosurfactant production by *Wickerhamomyces edaphicus*

Process parameter	Optimum level
Medium pH	6.0
Temperature	30 °C
Inoculum size	5 %
Inoculum age	72 h
Cottonseed oil concentration	4 % (v/v)
Yeast extract concentration	0.1 % (w/v)
Fermentation period	240 h

According to Table 1, *W. edaphicus* gave maximum biosurfactant production by using 4% cotton seed oil as a Carbon Source, peptone as a nitrogen source with medium pH 6.0 and incubation temperature 30°C for 240 h by providing 5% of inoculum of yeast culture.

4 Conclusion

In the present study, total 24 yeast strains were isolated from soil of mangrove ecosystem of Mundra, Kutch region of Gujarat. Based on the results of screening test, Wickerhamomyces edaphicus was selected for biosurfactant production. *W. edaphicus* gave highest oil displacement activity (4.0 cm) and emulsification index (62.96 %). The medium used for biosurfactant production by *W. edaphicus* contains cotton seed oil as a main carbon source, which was cheap as well as easily available in Gujarat, India. The study of different production process parameters showed that cottonseed oil 4 % (v/v) as a sole carbon source was found to be optimum. The optimum physical parameters such as pH and temperature for production was found to be 6.0 and 30°C respectively. Inoculum size of 5 % (v/v) was found optimum. Production medium with all above optimum parameter gives the highest production of biosurfactant at 10 days (240 h). The biosurfactant production in large scale by *W. edaphicus* will be studied further and industrial applications of purified biosurfactant can be tested.

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