Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 24, No. (1) : 2022 : 25-30

ISSN-0972-3005

http://doi.org/10.53550/AJMBES.2022.v24i01.005

EFFECT OF CARBON AND NITROGEN SOURCE ON PRODUCTION OF BIOSURFACTANT BY BACTERIAL SPECIES SPTSS1.

SHAIKH SHAGUFTA AND P V DHARANI

Department of Microbiology, Sheth P T Arts and Science College, Affiliated To Shri Govind Guru University, Godhra, Gujarat, India

(Received 17 October, 2021; Accepted 20 November, 2021)

Key words. *Biosurfactant, Hydrocarbons, Optimization*

Abstract– Biosurfacatant are surface active compound produced by some bacteria, yeast and molds as the part of their membrane structure. There are various types of microorganisms studied capable of producing biosurfactant by utilizing hydrocarbon compounds as source of carbon and energy. In this study biosurfactant producing organism was isolated from drained water left after boiling corn supplemented with ghee. Composition of culture medium optimized to increase the yield of biosurfactant production by isolated bacteria named as SPTSS1. Biosurfactant produced under optimized condition was characterized in terms of emulsification activity, drop collapse activity and oil displacement activity. Optimal medium for biosurfactant production was studied with different carbon and nitrogen sources.

INTRODUCTION

Utilization of hydrocarbon is a major source of environmental pollution. Leakage of oil storage tank, accidental spills, storage under improper condition leads to environmental contamination (Trindade *et al.,* 2005). Recent methods for remediation and degradation of hydrocarbon relies on utilization of chemicals. Nowadays bioremediation of pollutant is getting attention due to its low toxicity, cost effectiveness and ecofriendly characteristics compared to chemicals (Dhabi *et al*., 2020). Biosurfactant are surface active compound produced by some bacteria, yeast and mold as the part of their membrane structure. Range of microorganisms produces biosurfactant which can be lipopeptide, rhamnolipid, and glycolipid. Most commonly reported biosurfactant Lipopeptide that are known to be produced by *Bacillus* strain (Sarwar *et al.,* 2018). Biosurfactant are amphibolic in nature containing hydrophilic and hydrophobic ends capable of reducing surface tension by forming micelles which increases emulsification activity of biosurfactant against hydrocarbons (Luna *et al.,* 2013). For utilization of biosurfacatant in competition with chemical detergent low cost media is required for large scale production of biosurfactant. Factors affecting biosurfactant production are size of inoculum, pH, temperature, nutrient composition, ratio of carbon and nitrogen. Effect of carbon and nitrogen sources on production has been investigated by many researchers based on this studies carbon source is divided in water insoluble (hydrocarbons), and water soluble. Water insoluble carbon source produces more amount of biosurfactant compared to water soluble biosurfactant reported by (Banat *et al.,* 2014). In addition to carbon source, nitrogen source affects the yield and efficiency of biosurfactant production. It was reported that *Pseudomonas aeruginosa* preferred higher biosurfactant production byusing $NaNO_3$ as nitrogen source whereas *Bacillus subtilis* preferred ammonium nitrate (Arino *et al.,* 1996 and Abouseoud *et al.,* 2008). In this study biosurfactant producing organism is isolated from waste sample supplemented with ghee (clarified butter). The objective of this study were to know the effect of carbonand nitrogen sources on biosurfactant production and to get better yield of biosurfactant production by isolated organism.

MATERIALS AND METHODS

Sample collection

Drained water left after boiling corn was collected in

sterile glass bottle and supplemented with ghee (home made) as hydrocarbon source to isolate biosurfacatnt producing bacteria. Collected sample were brought to laboratory and stored at room temperature for one week.

Isolation and Characterization of biosurfactant Producing Bacteria

Corn water left after boiling corn was incubated at room temperature for two days and supplemented with ghee and further incubated for 7 days at room temperature. To isolate biosurfactant producing organism loop full of culture was streaked over sterile nutrient agar media (Hi madia) by random streaking method.

The inoculated plate was incubated at 37 °C for 24 hours. After 24 hours of incubation upon observation morphologically distinct colonies were observed on nutrient agar plate. Total six bacterial cultures were isolated from sample each culture was detected for hydrocarbon tolerance assay. SPTSS1 colony showing positive result for hydrocarbon tolerance assay further screened for biosurfacatant production activity by performing hemolytic activity, blue agar plate method, Modified Drop Collapse (MDC) assay, Sil Spreading Method (OSM) and Emulsification index (EI24%).

Screening method for biosurfactant producers

Tolerance against Hydrocarbon (TAH)

The test is performed according to method described by Satpute *et al.,* (2008). In order to analyze the ability of isolate to grow in presence of hydrocarbon, nutrient agar plate having isolated colonies were coated with seed agar followed by spreading of engine oil over surface. The plates were then incubated at 37 °C for 24 to 48 hours. A colony surrounded by emulsified halos was considered positive for biosurfactant production. Positive isolates were cultured in sterile medium containing tap water and engine oil as sole source of carbon.

Enrichment in broth

Positive isolates were inoculated in sterile broth prepared in Erlenmeyer flask composed of tap water and engine oil as hydrocarbon source (10ml tap water + 2 ml servo Engine oil) as sole source of carbon. Flask was incubated at room temperature for 7 days. After seven days incubation broth was observed for growth and degradation of hydrocarbon used as ingredient in broth.

Hemolytic activity (HA)

Loop full of bacterial culture from broth was streaked on sterile 5% blood agar plate and incubated at 37 °C for 24 to 48 hours. Hemolytic activity was detected as the presence of a definite clear zone around a colony (Carrillo *et al.,* 1996); Mulligan *et al.,* 1984; Rodrigues *et al.,* 2006.)

Blue agar plate method

Mineral salt agar medium supplemented with engine oil (2 ml), cetylpyridinum chloride and methylene blue was used for detection of anionic glycolipid. Wells were made using sterile cup borer on methylene blue agar plate loaded with fresh culture from broth. Followed by incubation at 37 °C for 24 to 48 hours (Siegmund *et al.,* 1991). A dark blue halo zone around the culture was considered positive for anionic biosurfactant production.

Modified Drop Collapse (MDC) assay

Screening of biosurfactant production was performed using the qualitative drop-collapse test (Batista *et al.,* 2005). This assay relies on the destabilization of liquid droplets by surfactants. Servo engine oil (containing 80% base oil, 1 to 10% alkenyl succinic compound, 1to 10% salicylic acid, base oil –petroleum and poly alpha olefins) was

Table 1. Effect of carbon source on biosurfactant production.

Carbon source (1%)	Peak (E24%)	OD AT 600 NM	Incubation Time(h)
Glucose	75%	0.08	96h
Maltose	40%	0.09	96h
Xylose	55%	0.04	96h
Servo engine oil	25%%	0.19	96h
Diesel	20%	1.13	168h
Glycerol	40%	0.28	96h
Banana peels	48%	1.83	240h
Potato peels	85%	1.79	288h
Sugarcane waste	50%	1.40	248h

applied as a coating layer on glass plate. A drop of incubated sample was placed over it to analyze drop collapse. Vim liquid soap was used as control.

Emulsification index (EI24%)

Equal volume of supernatant from incubated wastes and engine oil (Servo motor engine oil) in the ratio of (1:1) was taken in one test tube, and equal volume of supernatant from incubated wastes and paraffin oil in ratio of (1:1) were taken in another test tube. Contents of each tube were thoroughly mixed for 2 minutes using vortex mixer. The contents in the tubes were then left to stand for 24 hours at 37 °C. The same test was performed with vim liquid soap as a control. After 24 h, the emulsification activity was calculated using the following formula:

E24 (%) = total height of the emulsified layer/total height of the liquid layer.

Oil Spreading Method (OSM)

Oil spreading experiment was performed using the method described by Morikawa *et al.,* (2000) and Nayarisseri *et al.,* (2018). Ten ml distilled water added to Petri dish. To this, paraffin oil containing 1 ml of 0.5% Sudan black dye was added. This was followed by addition of 1 drop of incubated sample onto paraffin surface. The incubated sample containing biosurfactant separated the Sudan black dye containing paraffin layer and formed a clear zone. The same procedure was done with Vim liquid soap (Hindustan Unilever Limited, India) as a control. Vim liquid soap contains lauryl sulphate, disodium EDTA, lime juice, chlorine, and water.

Determination of anionic and cationic characteristics

Anionic or cationic property of biosurfacatant can be analyzed by using methylene blue and methyl orange dyes. Where cationic surfactant can be analyzed by using methyl orange dye and anionic biosurfactant can be analyzed by using methylene blue dye. To perform this test about 1 ml of sample was taken in test tube. Methylene blue was added in this tube to determine anionic characteristics and methyl orange was added in another test tube for cationic characteristics. Chloroform was added in each tube containing sample and dye after that all tubes were incubated at room temperature for 24 hours. After 24 hours of incubation tubes were observed for color change. Formation of yellow color indicates cationic character of biosurfactant and formation of blue color indicates anionic nature of biosurfactant.

Optimization procedure for biosurfactant production

Preparation of cell inoculums

Inoculum was prepared in broth containing 15 ml of distilled water supplemented with 2 ml of engine (Indian servo engine, 20w -40 MG) oil as carbon source. This was incubated at 37 °C for 48 hours. After incubation observed for bacterial growth and utilization of oil provided as carbon source.

Effect of different carbon and nitrogen sources on biosurfacatant production

Effect of carbon source on biosurfactant production

Production media was prepared according to Pandey *et al.,* (2014) in which 50 ml of distilled water was taken in ten 250 ml Erlenmeyer flask and autoclaved. 1% carbon source for example Glucose (lancer), fructose (S.d fine chem ltd., Biosar), xylose (Chemdyes corporation), servo engineoil (Indian servo engine oil 20w -40 MG), glycerol, diesel (obtained from B V Gandhi petrol pump), dried powder of banana peels (Prepared in working lab), dried powder of potato peel (Prepared in working lab) and dried powder of Sugarcane hulls (Prepared in working lab).1%v/v of 48h old inoculum was inoculated in each production media. They were then incubated at 37 °C for 24 hours. After 24 hours of incubation optical density was taken from each production media at 620 nm in colorimeter.

Effect of Nitrogen source on biosurfacant

Table 2. Effect of different nitrogen source on biosurfactant production.

Nitrogen source (1%)	Peak (E24%)	OD AT 600 NM	Incubation Time (h)
Urea	20%	0.14	48h
Peptone	60%	0.72	244h
Ammonium Sulphate	40%	0.21	24h
Ammonium chloride	50%	0.57	24h
Potassium Sulphate	48%	0.07	244h
Beef Extract	20%	1.56	168h

production

50ml of distilled water was taken in five 250 ml Erlenmeyer flask and autoclaved. 1% Nitrogen source tested were Urea (Chemdyes corporation), Peptone (Chemdyes corporation), Ammonium sulphate (S.D fine chem ltd., Biosar), Ammonium chloride (Sarabhai M chemicals, Baroda), Potassium nitrate (Sarabhai M chemicals, Baroda), and Beef Extract (Chemdyes corporation). 1% of 48h old inoculum was inoculated in each production media. They were then incubated at 37 °C for 24 hours. After 24 hours of incubation optical density was taken from each production media at 620nm using colorimeter

Biosurfcatant activity under optimized condition for each production media having different carbon and nitrogen source was evaluated by performing Emulsification activity at daily (24 h) interval with servo according to same procedure as described earlier.

RESULTS AND DISCUSSION

Results for isolation and Characterization of biosurfactant producing Bacteria

Colony of SPTSS1 isolates on nutrient agar was irregular in shape, wrinkled margin, dry, opaque, colorless, Gram positive short rods, arranged single and pairs. Among six colonies isolated on nutrient agar plate only one bacterial isolates named as SPTSS1 showed positive result for hydrocarbon tolerance assay. Organism inoculated in broth utilized engine oil as carbon source, pellicle growth of bacteria was observed over the surface of the broth after seven days of incubation period. Positive hemolytic activity was observed with complete zone of beta hemolysis with the diameter of 4.23cm diameter. Isolated bacterial culture was examined for blue agar assay produced dark blue halo around colony which was considered positive activity for biosurfactant production. Drop got collapsed after 1 min with the same diameter shown by vim. Oil got displaced immediately when cell free supernatnent dropped over oil surface

Results for effect of different carbon sources on biosurfactant production

Production of biosurfcatant was affected by different 1% carbon source like glucose, maltose. xylose, servo engine oil, glycerol, diesel, dried powder of banana peels, dried powder of potato peels, dried powder of sugarcane hulls. Result showed that production of biosurfactant was greater in production media containing potato peels and gassage compared to other carbon sources. Fig 1. Shows highest emulsification activity about 85% within 288 h of incubation was observed in production media containing potato peels as carbon source among ten tested production media.

Results for effect of different nitrogen sources on biosurfactant production

Production of biosurfcatant was affected by different 1% nitrogen sources like Urea, Peptone, Ammonium sulphate, Ammonium chloride, Potassium nitrate, and Beef Extract. Result showed that production of biosurfactant was greater in production media containing peptone, ammonium chloride, and potassium nitrate. Compared to other nitrogen sources. Fig. 2. Highest emulsification activity about 50% within shortest time after 24h of incubation was observed in production media of ammonium chloride among 6 tested production media.

DISCUSSION

In this study work was performed on one bacterial isolate named as SPTSS1. Total six organism isolated from sample screened by hydrocarbon tolerance assay from screening it was observed that SPTSS1 bacterial isolate was able to grow in presence of hydrocarbon sample, i.e. on agar plate coated with servo engine oil. Hemolytic activity is used as preliminary test to describe biosurfactant production as produced surfactant can lyse erythrocytes if biosurfactant is produced by organism. Factors affecting biosurfacant production like carbon and nitrogen source was considered for optimization. Production of biosurfactant with different carbon and nitrogen source was characterized based on bacterial growth, and emulsification activity (E24%). Biosurfactant exhibits the ability to enhance contact between oil and water which can be measured by emulsification activity. If the value of emulsification is high, i.e. contact between water and oil is also high. Emulsification activity (E24%) correlates with percentage of surfactant produced. Based on results of E24% and as shown in Figure 1 and 2 maximum amount of biosurfactant is produced in production media containing potato peels (E24%= 85%) indicates organism utilized it as best carbon source which is also observed in studies performed with

bacteria like *Pseudomonas aeruginosa* (Poonguzhal et al,). Potato peels is most cost effective waste product to be used as carbon source for biosurfactant production (Das and Kumar, 2018). Biosurfactant production by *Bacillus subtilis* and Streptomyces *spp.* has referred higher biosurfactant production with carbon source like date molasses (Naif Abdullah et al), (Korayem et al.,). Whereas in tested nitrogen sources higher biosurfactant production within shortest time was observed with ammonium chloride (E24%=50% within 24 hours). Other organism producing biosurfactant mostly utilize peptone as favored nitrogen source (Korayem et al,).

CONCLUSION

On the basis of screening isolated gram positive bacteria SPTSS1 gives the positive result for hydrocarbon tolerance assay, hemolytic test, oil spread test, emulsification activity, and drop collapse test for biosurfactant production. Biosurfacatnt production by isolated organism is affected by 1% carbon and nitrogen source. Based on result minimized source of C/N is potato peels / ammonium chloride is at 1:1 concentration.

REFERENCES

Abouseoud, M., Maachi, R., Amrane, A., Boudergua, S. and Nabi, A. 2008. Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. *Desalination.* 223 : 143–151.

Al-Dhabi, N.A., Esmail, G.A. and Valan Arasu, M. 2020. Enhanced Production of Biosurfactant from *Bacillus subtilis* Strain Al-Dhabi-130 under Solid-State Fermentation Using Date Molasses from Saudi Arabia for Bioremediation of Crude-Oil-Contaminated Soils. *Int. J. Environ. Res. Public Health.* 17 : 8446. https://doi.org/10.3390/ijerph17228446.

Arino, S., Marchal, R. and Vandecasteele, J.P. 1996. Identification and production of a rhamnolipidic biosurfactant by a *Pseudomonas* species. *Appl Microbiol Biotechnol.* 45 : 162. Avaialble:https://doi.org/10.1007/s0025300 50665 20.

Banat, I.M., Satpute, S.K., Cameotra, S.S., Patil, R. and Nyayanit, N.V. 2014. Cost effective technologies and renewable substrates for biosurfactants' production. *Front Microbiol.* 5 : 697. DOI: 10.3389/fmicb.2014.00697

Batista S.B. A, Mounteer, A.H., Amorim, F.R. and T´otola, M.R. 2005. Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites.

Carrillo, P.G., Mardaraz, C. and Pitta-Alvarez, S.I. 1996. Isolation and selection of biosurfactant-producing bacteria. *World J Microbial Biotechnology*. 12 : 82–84. https://doi.org/10.1007/BF00327807

Das, A.J. and Kumar, R. 2018. Utilization of agro-industrial waste for biosurfactant production under submerged fermentation and its application in oil recovery from sand matrix. *Bioresource Technology*. 260 : 233-240. https://doi.org/10.1016/j.biortech.2018.03.093

Korayem, A.S., Abdelhafez, A.A., Zaki, M.M. and Saleh, E.A. Optimization of biosurfactant production by Streptomyces isolated from Egyptian arid soil using Plackett–Burman design.

Luna, J.M., Rufino, R.D., Sarubbo, L.A. and Campos-Takaki, G.M. 2013. Characterization, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candidasphaerica* UCP0995 for application in the petroleum industry. *Colloids Surf. B* 102 : 202–209. [CrossRef].

Morikawa, M., Hirata, Y. and Imanaka, T. 2000. A study on the structure-function relationship of lipopeptide biosurfactants. *Biochim Biophys Acta.* 1488(3) : 211-218. doi: 10.1016/s1388-1981(00)00124-4. PMID: 11082531.

Naif Abdullah Al-Dhabi, Galal Ali Esmail and Mariadhas Valan Arasu, Enhanced Production of Biosurfactant from *Bacillus subtilis* Strain Al-Dhabi-130 under Solid-State Fermentation Using Date Molasses from Saudi Arabia for Bioremediation of Crude-Oil-Contaminated Soils.

Nayarisseri, A., Singh, P. and Singh, S.K. 2018. Screening, isolation and characterization of biosurfactant producing *Bacillus subtilis* strain ANSKLAB03. *Bioinformation.* 30; 14(6) : 304-314. doi: 10.6026/97320630014304. PMID: 30237676; PMCID: PMC6137570.

Pandey, M. Mishra, and Rai, V. 2014. Optimization and Characterization of Biosurfactant Producing Microbes and Expression of Biosurfactant Producing Genes in Non Biosurfactant Producing Miocrobes" *Int. J. of Pharm. Res. & All. Sci.* 3(1) : 47-56.

Poonguzhali*1, P., Rajan1, S. and Parthasarathi2, R. Utilization of Potato peel and Curd whey as the Agro industrial substrates for Biosurfactant production using *Pseudomonas aeruginosa.* PBS29.

Rodrigues, L.R., Teixeira, J.A., van der Mei, H.C. and Oliveira, R. 2006. Isolation and partial characterization of a biosurfactant produced by *Streptococcus thermophilus* A. *Colloids Surf B Biointerfaces.* 1; 53(1) : 105-12. doi: 10.1016/j.colsurfb.2006.08.009. Epub 2006 Aug 22. PMID: 16987640.

Sarwar, A., Brader, G., Corretto, E., Aleti, G., Abaidullah, M., Sessitsch, A. and Hafeez, F.Y. 2018. Qualitative analysis of biosurfactants from *Bacillu*s species exhibiting antifungal activity. *PLoS ONE.* 13, e0198107.

Satpute, S. K., Bhawsar, B.D., Dhakephalkar, P. K, Chopade, B.A. 2008. 2008. Assessment of different screening methods for selecting biosurfactant producing marine bacteria. *Indian J Marine Sci.* no.

37 : 243–250.

Siegmund, I. and Wagner, F. 1991. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. *Biotechnol Tech.* 5 : 265–268.

Trindade, P., Sobral, L., Rizzo, A., Leite, S. and Soriano, A. 2005. Bioremediation of a weathered and a recently oil-contaminated soils from Brazil: A comparison study. *Chemosphere.* 58 : 515–522. [CrossRef] [PubMed].

Vanessa Walter, Christoph Syldatk, and Rudolf Hausmann, Screening Concepts for the Isolation of Biosurfactant Producing Microorganisms. *Landes Bioscience.* 2000-2013.