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Evaluation of the effect of various nutritional and environmental factors on biosurfactant production by *Staphylococcus epidermidis*

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Abstract. *Alyousif NA, Al-Tamimi WH, Al-sahib MAA. 2022. Evaluation of the effect of various nutritional and environmental factors on biosurfactant production by* Staphylococcus epidermidis. *Biodiversitas 23: 3533-3538.* Biosurfactants are biological surface-active compounds synthesized mainly by hydrocarbon-utilizing bacteria. The properties of biosurfactants make them promising compounds for application in various fields. The current study evaluated the effect of various nutritional and environmental factors on biosurfactants produced by *Staphylococcus epidermidis*. This bacterium, for first time in world was isolated from oilfield reservoir in the current study and identified by 16S rDNA sequencing and considered biosurfactant producer according to screening tests. Several factors were evaluated in the current study to assess the optimal conditions for producing the biosurfactant. The results demonstrated that the best carbon source was olive oil with 2% concentration and glutamic acid was the best nitrogen source with 0.2% concentration. The study demonstrated that the best biosurfactant production was recorded at incubation time of 5 days with a 3% inoculum size. Further study is required to determine the chemical structure of produced biosurfactants and to evaluate the potential application of biosurfactants in various fields, therefore constituting a stimulus for future studies.

Keywords: Biosurfactant, carbon and nitrogen sources, environmental factors, *Staphylococcus epidermidis*

INTRODUCTION

Biosurfactants (BSs) are surface-active biomolecules synthesized mainly by hydrocarbon-utilizing organisms such as bacteria and fungi, which are released into the environment or adhered to the producer cell's surface (Ndlovu et al. 2016; Alyousif et al. 2020a). BSs are amphiphilic compounds containing both hydrophobic and hydrophilic groups that solubilize two separate phases in a heterogeneous solution by interacting with the interface between them (Mani et al. 2016). Oil sludge bacteria have potential as biosurfactants because they can produce glycolipid compounds (Sari et al. 2015). According to chemical structures, they exhibit a broad diversity such as phospholipids, glycolipids, lipoproteins, fatty acids, particulate and polymeric biosurfactants (Aparna et al. 2012). BSs have different functional properties, including wetting, cleansing, emulsification, foaming, surface activity and anti-microbial activity, in addition to the ability to function in severe conditions like high pH, temperature and salt concentration, making them suitable for use in pharmaceuticals, cosmetics, environmental remediation agriculture, food industries and enhanced oil recovery (Sachdev and Cameotra 2013; Elazzazy et al. 2015).

Biosurfactant-producing bacteria are widespread in hydrocarbon-contaminated natural habitats such as soil, water, and production water of oil reservoirs (Sohail and Jamil 2020). Different genera of bacteria are able to produce different forms of biosurfactants. The species of *Pseudomonas*, *Rhodococcus*, *Lactobacillus*, *Bacillus*, *Acinetobacter*, *Arthobacter* and *Candida* are most genera utilized for biosurfactant production (San Keskin et al. 2015).

The bacterial genus *Staphylococcus* is a known for its pathogenic effect on humans and animals, but some *Staphylococcus* strains isolated from natural environments could produce secondary metabolites that exhibited biotechnological significance (Nair et al. 2014). Some *Staphylococcus* spp. revealed the ability to produce various secondary metabolites that can be used in variety of fields. Different species of *Staphylococcus* such as *S. aureus*, *S. epidermidis*, *S. hominis*, *Staphylococcus* sp. strain 1E, *S. saprophyticus*, *S. xylosus* demonstrated the ability of biosurfactant production (Eddouaouda et al. 2011; Hamed et al. 2012; San Keskin et al. 2015; Mani et al. 2016). *Staphylococcus epidermidis* is a normal bacteria inhabitant of human skin. It causes nosocomial infections, especially in immunocompromised patients or those suffer from injuries (Eladli et al. 2018). *Staphylococcus epidermidis* isolated from seawater was found capable of producing biosurfactant for the first time (Hamed et al. 2012).

The current study aimed to evaluate the effect of various nutritional and environmental factors, including different carbon sources, nitrogen sources, inoculum size and incubation period on biosurfactant production by *S. epidermidis*, which is isolated from crude oil of oilfield.

MATERIALS AND METHODS

Isolation and identification of bacterial sample

The bacterium utilized in present study was isolated in previous study (Alyousif et al. 2020a) from crude oil of Al-Garraf oilfield in Thi-Qar province (31°14′N 46°19′E). The bacterium was identified by molecular technique using sequence analysis of 16S ribosomal DNA gene. The bacterial DNA was isolated by using bacterial DNA isolation kit manufactured by the Geneaid company. The PCR technique was used to amplify the 16S ribosomal DNA gene by using the bacterial forward primer 27F (5-AGAGTTTGATCCTGGCTCAG-3) and reverse primer 1492R (5-GGTTACCTT GTTACGACTT-3). The reaction was prepared in tube with a total volume of 50 µl of PCR components. The first PCR cycle named denaturation, was carried out at 96°C (3 min), then 27 cycles as follow of 96°C (30 s), 56°C (25 s) and 72°C (15 s) and final cycle was extension for 10 min at 72°C (Miyoshi et al. 2005). The products of PCR reaction were sequenced by Macrogen Company (Korea). The 16S ribosomal DNA gene sequence was proofread and aligned with bacterial 16S rDNA database available at NCBI using BLAST to determine the similarity and identification of the isolated bacterium.

Preparation of the bacterial inoculum

The bacterium was inoculated in a 250 mL flask containing 50 mL of autoclaved nutrient broth (Himedia, India) and incubated for 24 h at 30°C. The minimal salt medium was inoculated with 5% (v/v) of prepared inoculum.

Biosurfactant production

The minimal salt medium was taken from (Deng et al. 2014) and contained the following ingredients (g/l), including Na_2HPO_4 (3), KH_2PO_4 (2), NH_4NO_3 (1), $MgSO_4.7H_2O$ (0.7), NaCl (5) and 1 mL /l trace salt solution. The trace salt solution containing (mg/l) $CuSO_4$ (0.5), $FeCl_3$ (30), $MnSO_4.H_2O$ (0.5), $CaCl_2$ (20) and $ZnSO_4.7H_2O$ (10). One hundred mL of minimal salt medium was prepared and autoclaved in a 250 mL flask at 121°C for 15 min, then the minimal salt medium was inoculated with 5 mL (10^6 CFU/mL) of an activated inoculum. The flask was incubated for 7 days at 30°C with agitation (150 rpm/min) in a shaking incubator.

Screening for producing the biosurfactant

After 7 days of incubation time, the cultures broths were centrifuged at 6000 rpm to eliminate the bacterial cells for 15 min at 4°C (Nayarisseri et al. 2018). The bacterial cell-free supernatants were subjected to the following tests to select the optimum factors for biosurfactant producing.

Hemolytic assay

The hemolytic assay was used to examine the ability of bacterium to synthesize biosurfactant according to the protocol described by Youssef et al. (2009). The bacterium was streaked on the plate of 5% human blood agar with incubation time of 24 h at 30°C, then the hemolysis zone was observed around the spot.

Oil spreading assay

The assay was performed by pouring 40 mL of distilled water into a Petri dish with a 15 cm diameter, then pouring 20 mL of crude oil onto the water surface to form a thin layer. 10 µL of supernatant was poured onto oil surface. The diameter of the clearing zone was calculated and compared with water as a control (Satpute et al. 2010).

The emulsification index

Two mL of both bacterial cell-free supernatant and kerosene were mixed and vortexed for 2 min. After a 24 h incubation period at room temperature, the emulsion layer height was measured to calculate the emulsification index (Tao et al. 2020). The emulsification index (E24%) is measured by the following equation:

$$E24\ (\%) = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100\%$$

Emulsification activity

A total of 0.5 mL of the supernatant, 7.5 mL of Tris-Mg [20mM Tris HCl (pH 7.0) and 10 mM MgSO4] and 0.1 mL of kerosene were mixed together in one tube. The mixture was vortexed for 2 min and left for 1 h. The samples' absorbance was measured at 540 nm, the optical density was used to calculate emulsification activity (EA) (Sifour et al. 2005).

Biomass determination

The cultures broth was centrifuged at 5000 rpm to separate the biomass for 15 min at 5°C and then oven-dried the pellet at 105°C for 24 h (Santos et al. 2018). The incubation period of the flask was at 30°C with agitation (150 rpm/min) in a shaking incubator.

Selection of factors affecting biosurfactant production

The culture media was incubated under different nutritional and environmental conditions to choose the best nutritional and environmental factors for producing the biosurfactant, which included the following factors: (source of carbon, concentration of selected carbon, source of nitrogen, concentration of selected nitrogen, incubation period and inoculum size), after that the production of biosurfactant was assessed by testes of emulsification index, oil spreading and emulsification activity. Total of 100 mL of production medium supplemented with 1% of one of the following sources of carbon, including glucose, corn oil, olive oil, glycerol, lactose, mannitol, and sunflower oil, was examined to choose the best source of carbon. The incubation time of the medium for 7 days at 30°C in a shaking incubator. Five concentrations of olive oil were tested, including the following concentrations (1, 1.5, 2, 2.5, and 3%) to select the best carbon source concentration. Four nitrogen sources (glutamic acid, KNO_3, NH_4NO_3 and urea) were tested to choose the optimum nitrogen source in production medium. Four increasing concentrations of glutamic acid (0.1%, 0.2%, 0.4% and 0.6)

were tested to determine the optimum concentration of nitrogen. The inoculum volume was evaluated by using different inoculum volumes (1, 2, 3, 4 and 5%) of the bacterium. The incubation period for producing the biosurfactant was evaluated by incubating the medium at various incubation periods, including 1, 2, 3, 4, 5, 6, and 7 days.

Statistical analysis

The differences among the factors were assessed using one-way ANOVA of SPSS software (version 20). P-value less than 0.05 was regarded as statistically significant. The average of emulsification activity and biomass values was calculated and expressed as mean standard deviation.

RESULTS AND DISCUSSION

Identification of bacterial isolate

The isolate was identified by PCR technique to amplify 16S ribosomal DNA gene. The PCR product was observed on agarose gel electrophoresis under UV transilluminator at the position of nearly 1500 bp in comparison with the DNA ladder as shown in figure 1. The sequence analysis of 16S ribosomal DNA gene of the isolate identified this isolate as *S. epidermidis*.

Screening for biosurfactants production

Staphylococcus epidermidis was screened to assess the ability of isolate to produce biosurfactants by using screening tests. For the hemolytic test, the bacterium *S. epidermidis* revealed a clear zone around the colony of bacterium on blood agar and considered positive results, as shown in (Figure 2A). The results showed a 53.3% value for emulsification index test (Figure 2B), 10 cm diameter of clear zone for the oil spreading test (Figure 2C) and 0.896±0.082 for emulsification activity therefore, they considered positive results for producing biosurfactant. The biomass concentration obtained from *S. epidermidis* was 2.237±0.080 g.

Screening of nutritional and environmental conditions for enhanced the production of biosurfactants

Several factors were evaluated in the present study to determine the best conditions for producing biosurfactants by *S. epidermidis*. Various sources of carbon were evaluated for producing biosurfactants. The results presented in Table 1 demonstrated that best source of carbon for producing the biosurfactant was olive oil with emulsification activity (0.896± 0.055), E24% (53.3%), oil spreading fourteen cm and biomass 2.237± 0.035 gm. Mannitol was the weakest source of carbon for producing the biosurfactant with emulsification activity (0.043± 0.121), no value recorded for E24%, oil spreading 0.3 cm and biomass 1.971± 0.072 gm.

Different olive oil concentrations were assessed as carbon sources for producing the biosurfactant by *S. epidermidis*. The results given in table 2 demonstrated the best concentration of olive oil. The 2% concentration of olive oil gave the optimum results for emulsification activity (1.240± 0.063), E24% (60%), oil spreading 14 cm and biomass 2.331± 0.084 gm with significant differences from other concentrations ($P \leq 0.05$).

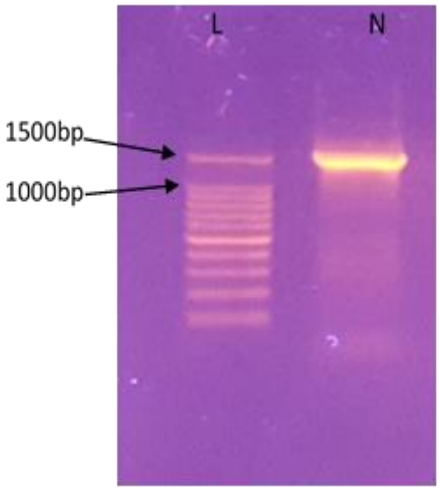
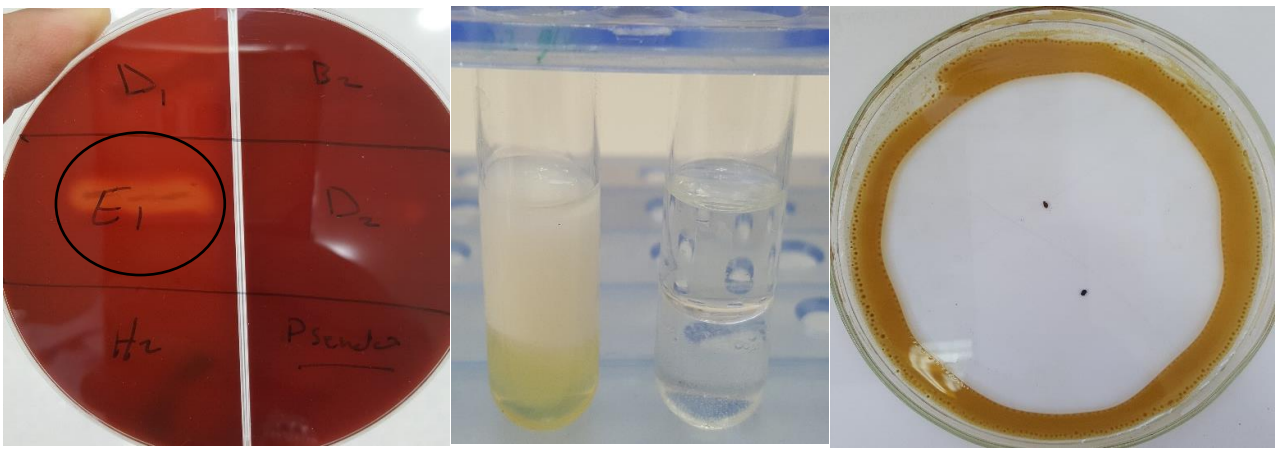


Figure 1. The PCR product of 16S ribosomal DNA gene. Lane L: 100 bp DNA ladder. Lane N: 16s rDNA gene



A **B** **C**

Figure 2. Screening tests of biosurfactant production A. Hemolytic assay B. emulsification index, C. oil spreading test

Table 1. The influence of different carbon sources on production of biosurfactants by *Staphylococcus epidermidis*

Carbon sources	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/cm	Biomass g/L
Olive oil	0.896± 0.055*	53.3	14	2.237± 0.035
Sunflower oil	0.497± 0.036	36	9.5	1.326± 0.063
Corn oil	0.111± 0.073	3.3	5	1.413± 0.321
Glycerol	0.251± 0.216	26.6	7	1.661± 0.064
Lactose	0.045± 0.053	nil	0.2	1.317± 0.096
Glucose	0.044± 0.072	nil	0.3	1.825± 0.083
Mannitol	0.043± 0.121	nil	0.3	1.971± 0.072

Table 2. The influence of olive oil concentration on production of biosurfactant by *Staphylococcus epidermidis*

Olive oil (%)	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass g/L
1	0.902± 0.061*	53.3	14	2.425± 0.042
1.5	1.015± 0.034	53.3	14	2.667± 0.052
2	1.240± 0.063	60	14	2.331± 0.084
2.5	1.138± 0.620	53.3	14	2.173± 0.062
3	0.823± 0.083	50	14	2.215± 0.033

Note: * Mean ± SD, n=3, $P \leq 0.05$

Table 3. The influence of different nitrogen sources on production of biosurfactants by *Staphylococcus epidermidis*

Nitrogen sources	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass g/L
Glutamic acid	1.446± 0.038*	60	14.5	2.651± 0.075
KNO_3	1.323± 0.034	60	14	2.723± 0.042
NH_4NO_3	1.257± 0.051	60	14	2.425± 0.041
Urea	1.020± 0.214	53.3	14	2.342± 0.085

Note: * Mean ± SD, n=3, $P \leq 0.05$

Table 4. The influence of Glutamic acid concentration on production of biosurfactant by *Staphylococcus epidermidis*

Glutamicacid concentration (%)	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass g/L
0.1	1.325± 0.061*	60	14.5	2.551± 0.072
0.2	1.423± 0.112	60	14.5	2.624± 0.038
0.4	1.587± 0.036	60	14.5	2.535± 0.092
0.6	1.302± 0.081	60	14.5	2.723± 0.022

Note: * Mean ± SD, n=3, $P \leq 0.05$

Table 5. The influence of inoculum size on production of biosurfactant by *Staphylococcus epidermidis*

Inoculum size (%)	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass g/L
1	1.503± 0.061*	60	14.5	2.517± 0.034
2	1.578± 0.201	60	14.5	2.821± 0.022
3	1.621± 0.033	60	15	2.923± 0.083
4	1.523± 0.071	60	14.5	2.512± 0.502
5	1.417± 0.095	60	14.5	2.624± 0.0104

Note: * Mean ± SD, n=3, $P \leq 0.05$

Table 6. The influence of incubation time on production of biosurfactant by *Staphylococcus epidermidis*

Incubation period	Emulsification activity/ 540 nm	Emulsification index (E24%)	Oil spreading/ cm	Biomass g/L
1	0.186 ± 0.016*	3.3	4	0.374 ± 0.071
2	0.491 ± 0.063	26.6	8	0.873± 0.082
3	0.885 ± 0.221	53	10	1.943± 0.063
4	1.286 ± 0.036	60	14	2.412± 0.502
5	1.678 ± 0.052	60	15	2.622± 0.015
6	1.435 ± 0.027	60	14.5	2.853± 0.720
7	1.387 ± 0.241	60	14	2.752± 0.045

Note: * Mean ± SD, n=3, $P \leq 0.05$

Various nitrogen sources were assessed as sole nitrogen sources for producing the biosurfactant by *S. epidermidis*. The results shown in table 3 depicted that Glutamic acid was the best source of nitrogen for producing the biosurfactant with values (1.446± 0.038, 60%, 14.5 cm and 2.651±0.0752 gm) for emulsification activity, E24%, oil spreading and biomass respectively with significant differences of nitrogen sources ($P \leq 0.05$). Urea was found to be the weakest source of nitrogen for producing the biosurfactant with emulsification activity (1.020± 0.214), E24% (53.3), oil spreading 14cm and biomass 2.342± 0.085 gm.

Several concentrations of Glutamic acid were assessed as a sole source of nitrogen for producing the biosurfactant by *S. epidermidis*. The results given in table 4 demonstrated the best concentration of Glutamic acid. The concentration of 0.4% of Glutamic acid gave the optimum results with values (1.587± 0.036, 60%,14.5 cm and 2.535± 0.092 gm) for emulsification activity, E24%, oil spreading and biomass, respectively with significant differences of other concentrations of Glutamic acid ($P \leq 0.05$).

Several sizes of inoculum were examined to assess the best inoculum size for biosurfactant production by *S. epidermidis*. The results provided in table 5 demonstrated that the inoculum size above 3% had no significant effect on biosurfactant production. The concentration of 3% of inoculum size gave the best finding with values (1.621± 0.033, 60%, 15 cm and 2.923± 0.083 gm) for emulsification activity, E24%, oil spreading and biomass respectively with significant differences of other concentrations of inoculum size ($P \leq 0.05$).

Several incubation periods were examined to determine the optimum incubation period suitable for producing the biosurfactant by *S. epidermidis*. The results provided in table 6 demonstrated that the incubation period of 6 days gave the best findings with values (1.678 ± 0.052, 60%, 15 cm and 2.622± 0.015 gm) for emulsification activity, E24%, oil spreading and biomass along with significant differences of another incubation period ($P \leq 0.05$). The values of screening tests were raised by raising the incubation days to five, but the values of screening tests decreased at 6 and 7 days of incubation.

Discussion

The present study was carried out to identify biosurfactant producing bacteria from crude oil oil in the oilfield and evaluate the effects of nutritional and environmental factors on biosurfactant production. The molecular identification revealed the identity of bacterial isolate as *S. epidermidis*. The majority of *Staphylococcus* sp. is known as pathogen for humans and animals. Several *Staphylococcus* strains isolated from many natural environments could be used for producing substances with high values for biotechnological application (Eddouaouda et al. 2011).

The screening tests used to evaluate biosurfactant production were emulsification index test, oil spreading test, hemolytic assay and emulsification activity, as these tests are simple and quick for screening and predicting biosurfactant production by bacteria. The hemolytic assay is used as primary screening method for detecting the ability of bacteria to synthesize biosurfactants. Ibrahim (2016) observed that some biosurfactants can cause hemolysis but that the hemolysis is not limited to biosurfactants and hemolysis could also be done by bacterial lytic enzymes**.**

Emulsification index is an important test to select the potential producers of biosurfactants. The principle of emulsification index test, as well as oil spreading test and emulsification activity, is based on lowering the interfacial tension of liquids and the ability of biosurfactants to emulsify, independent of their structure (Alyousif et al. 2020b). The value of emulsification index and oil spreading test refers to the amount of biosurfactant produced by bacterial isolate.

The screening tests demonstrated that the optimum carbon source was olive oil, with a 2% concentration for producing the biosurfactant. *Staphylococcus epidermidis* strain was isolated from crude oil, and thus, the substrate formed a natural carbon source for its natural environment.

The ability of *S. epidermidis* to use olive oil as a carbon source rather than other compounds for biosurfactant production is due to the strain's ability to synthesize lipase, which aids in the absorption of fatty acids found in olive oil. The growth of bacteria on carbohydrate substrates, which causes a decrease in the medium pH and thus inhibits biosurfactant production (Chandra et al. 2020). The same results demonstrated by Eddouaouda et al. (2011) that the best carbon source for producing biosurfactant was olive oil by *Staphylococcus* sp. strain 1E, among the other tested sources.

The current study revealed that glutamic acid was the optimal nitrogen source with a 0.2% concentration for biosurfactant production. The bacteria need nitrogen sources for synthesizing enzymes essential for their survival and metabolic processes (Okoliegbe and Agarry 2012). Hu et al. (2015) demonstrated that the biosurfactant was optimally produced by *Vibrio* sp. by using 1.1% yeast extract as nitrogen source. While Fazli and Hertadi (2018) showed rhamnolipid production by *P. aeruginosa* when it used 0.2% $NaNO_3$ as the nitrogen source.

The results also revealed that the maximum biosurfactant production was recorded at 3% of inoculum size. Alyousif et al. (2020b) reported the optimal size of inoculum for highest production of rhamnolipid biosurfactant from *P. aeruginosa* was 3%. While Keskin et al. (2015) reported the optimal size of inoculum for highest production of biosurfactant from *S. xylosus* was 5%. The current study demonstrated the maximum biosurfactant production at 5 days of incubation period. The increasing incubation period led to the intervention between bacterial metabolites and biosurfactants, which may lead to decreased biosurfactant activity. Mani et al. (2016) reported that biosurfactant produced by *S. saprophyticus* SBPS 15 increased with incubation period and reached maximum production after 66 h of incubation period. The obtained results during the current study constitute a stimulus for future studies to determine the chemical structure of produced biosurfactant, evaluate the potential

antibacterial activity of biosurfactant and determine the possibility of using it in the bioremediation of pollutants.

In conclusion, the present study revealed that *S. epidermidis* isolated from crude oil of oilfield reservoir for first time was efficient biosurfactant producing bacterium. The bacterium was evaluated for several factors to determine the optimal conditions for producing of biosurfactant. The finding revealed that optimum conditions for producing of biosurfactant were olive oil (2%), glutamic acid (0.2%), 5 days incubation period and 3% inoculum size.

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