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Production of Microbial Biosurfactants and their Use in the Biorremediation of Soils Contaminated with Polycyclic Aromatic Hydrocarbons

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***Abstract*— Bioremediation is a process in which microorganisms are used for partial or total decomposition of environmental pollutants, among which polycyclic aromatic hydrocarbons (PAHs) have received great attention due to their toxicity. Biosurfactants are low toxicity and biodegradable active tenside compounds that have the potential to degrade pollutants mainly in petroleum products. In this context, the present work aimed to produce biosurfactants from the bacteria Bacillus subtilis CCT 7678 and Corynebacterium aquaticum and to use them in the bioremediation of soils contaminated with petroleum and fuel oil. The submerged aerobic culture of the bacterium Bacillus subtilis CCT 7678 was carried out in 1000 ml Erlenmeyer type fermenters and the solid state culture of Corynebacterium aquaticum was carried out in fixed bed column bioreactors with internal dimensions 50 x 250 mm (diameter and height). In both soil contaminated with oil and soil contaminated with fuel oil, the highest rates of PAH degradation (97.3 and 93.5%, respectively) were obtained with the biosurfactants of the bacterium Corynebacterium aquaticum, which was characterized by a reduction of Surface tension (72 mN m^{-1} to 30.4 mN m^{-1}) and emulsifying activity 99.7%.**

***Keywords*— Bacillus subtilis, Biosurfactant, Corynebacterium aquaticum, Emulsifier, PHA**

I. Introduction

Global industrialization is based on the energy capacity of oil and its derivatives. This increases the possibility of contamination by unusual spills, generally reaching large land, sea, lake and other areas. Several followers, one of them being bioremediation, which allows the use of microorganisms for the degradation of these compounds, most of them highly harmful to human health [1], have studied the use of new technologies aimed at minimizing the effects of these contaminants.

The biodegradation of hydrocarbon rich compounds is dependent on their chemical structure and the size of their carbon chain, the lower the chain the more soluble and the faster their degradability.

The biosurfactants interact with the hydrocarbons and thus, make them more soluble in water, which increases the availability of the contaminants to the microorganisms and consequently their metabolization [2].

Biosurfactants are compounds produced by several species of microorganisms that are characterized by their surfactant activity. These compounds comprise a variety of chemically different molecules such as glycolipids, liposaccharides, phospholipids, fatty acids and neutral lipids, lipopeptides [3-6].

Most of the surfactants used commercially are synthesized from petroleum derivatives. However, the increased environmental concern combined with the new environmental control legislation has led to the search for biological surfactants as a result of being biodegradable, thus reducing the impact Environmental [7,8]. Among the most effective biosurfactants are lipopeptides produced by bacteria of the genus Bacillus, especially those produced by Bacillus subtilis [9]. In addition to the genus Bacillus, the bacterium Corynebacterium alkanolyticum studied by Crosman, Pinchuk and Cooper [10] produced phospholipid-type biosurfactants capable of reducing surface tension to 32 mN m-1. Bognolo [3] reports that biosurfactants produced by Corynebacterium and B. subtilis have shown promising results in the removal of tar in contaminated areas.

In this way, the present work proposed to cultivate bacteria Bacillus subtilis CCT 7678 and Corynebacterium aquaticum, producing biosurfactants for bioremediation of soils contaminated with petroleum and fuel oil.

II. Material And Methods

A. Growth and maintenance of microorganisms

The microorganisms used in this study were Bacillus subtilis CCT 7678 and Corynebacterium aquaticum. The André Tosello Foundation located in Campinas - SP, Brazil, donated the lineage of Bacillus subtilis CCT 7678.

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The Food Microbiology Laboratory of the Faculty of Food Engineering of the State University of Campinas (FEA/UNICAMP) supplied the bacterium Corynebacterium aquaticum.

Bacterial cultures were maintained under refrigeration in test tubes and Erlenmeyer's containing nutrient agar, which was formulated as follows (g L^{-1}): meat extract (1.0); Yeast extract (2.0); Peptone (5.0); NaCl (5.0) and agar (15.0) [11].

The inoculum propagation was carried out in Erlenmeyer flasks containing nutrient agar, formulated as follows (g $^{L-1}$): meat extract (1.0); Yeast extract (2.0); Peptone (5.0); NaCl (5.0). Cultures were incubated for 48 h at 30 °C. After scraping the agar surface with nutrient medium and reaching the optical density 0.8-0.9 at 600 nm wavelength, the suspensions were used as inocula and added to the culture medium in the concentration of 2% (v v^{-1}) [11].

B. Solid state fermentation

Corynebacterium aquaticum was cultured in solid state in fixed bed column bioreactors with internal dimensions 50 x 250 mm (diameter and height) for 144h. The compressed air used first passed through filters filled with glass wool and later its flow was controlled by previously calibrated parameters (Cole-Parmer Instrument Company, USA). The air stream was humidified, and then the droplets were removed and then injected into the columns. The incubation temperature of the medium was maintained constant at 30 °C by circulating water (from thermostated bath) through the bioreactor jacketed. The material used was sterilized at 121 °C for 15 min. Humidity was set at 65% [12].

The culture medium was composed of rice husk and wheat bran (used as carbon source) in proportions 15 and 85%, respectively, with addition of nutrient solution composed of: NH_4NO_3 (50.0 mM), Na_2HPO_4 (3.0 mM), KH_2PO_4 (3.0 mM), $CaCl_2$ (7.0 µM), $MgSO_4.7H_2O$ (0.8 mM), sodium EDTA (4.0 µM), and $FeSO_4.7H_2O$ (2.0 mM) [13]. The wheat bran used was retained in Tyler 35 and 32 sieves, opening between 0.42 and 0.50 mm, respectively [12].

The wheat bran used as a carbon source was supplied by Moinho do Sul S/A located in Rio Grande, Rio Grande do Sul, Brazil. The rice husk was supplied by the Casa do Arroz sugar mill, Santa Vitória do Palmar, Rio Grande do Sul, Brazil.

C. Submerged fermentation

The submerged culture of the bacterium *Bacillus subtilis* CCT 7678 was carried out in 1000 ml Erlenmeyer type fermentors with a 500 ml usable volume at 30 ºC, orbital agitation (Model Certomatr BS-1 B Incubator - Braum Biotech International, Germany) at 200 rpm for 72 h. The mineral medium was formulated as described by Yeh, Wei and Chang [13], consisting of NH_4NO_3 (50.0 mM), Na_2HPO_4 (3.0 mM), KH_2PO_4 (3.0 mM), $CaCl_2$ (7.0 µM), $MgSO_4.7H_2O$ (0.8 mM), sodium EDTA (4.0 µM), and $FeSO_4.7H_2O$ (2.0 mM). The carbon source used was 4% glucose and the initial pH of the medium was 7.0. After the culture medium was sterilized at 121 °C for 15 min.

D. Extraction of biosurfactants

The extraction of the surfactants produced by Corynebacterium aquaticum was carried out with water at 90 °C in the proportion 1:9 (fermented meal:solvent) [14]. After addition of the solvent, the sample was subjected to stirring (Model Certomatr BS-1 B Incubator - Braum Biotech, Germany) at 160 rpm and 50 ºC for 30 min, then vacuum filtered to remove excess solids Gifts. The filtrate was centrifuged (Centrifuge model CR 22GIII - Hitachi, Japan) at 9200 x g for 15 min. The supernatant was collected and used for the bioremediation process [15].

When the biomass was generated by the bacterium *Bacillus subtiis* CCT 7678, the extraction of surfactants was carried out after 72 h of submerged fermentation. The medium was filtered on quantitative filter paper (JP) followed by sterilization at 121 °C for 15 min.

E. Determination of surface tension

The surface tension of the extract was determined on a tensiometer (Kruss Processor Tensiometer K-9, Germany), using the Du Nouy ring method. This tensiometer determines surface and interfacial tensions with the help of a suspended ring and fixed in a precision scale. The sample, always liquid, was placed in a specific container of the equipment, the ring was immersed in the liquid and zeroed. The ring was then lowered so that the liquid film produced below the ring was elongated. When the liquid film was stretched, the maximum force was determined and measured, obtaining the surface tension. The surface tension was determined with the sample in contact with the air [16, 17].

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F. Emulsifying activity

The water-in-oil (AEo/w) emulsifying activity of the extract was determined according to the method described by Cooper and Goldenberg [18]. The extract containing the biosurfactants was homogenized with sunflower oil with the aid of a tube agitator (model AP 56 -Phenix, Brazil) and kept at rest for 24 h. The emulsifying activity was calculated by Equation 1.

$$EA\ (\%) = \frac{\text{height of the emulsion} \times 100}{\text{height total}} \quad (1)$$

G. Treatment of data

The results of surface tension and emulsifying activity were evaluated by means of the difference of averages (Tukey's test) at a significance level of 5%, in relation to the different biosurfactants produced.

H. Application of biosafeters in the process of biorremediation of soil contaminated with hydrocarbons

Collection of soil sample

The sandy soil was collected in the locality of Arraial, Rio Grande (RS) with the following locations, latitude 29º50'043 "S and longitude 50º31'216" W. Sieves of 4.00 to 0.062 mm were used and the mass retained in each of the sieves was compared to the Wentworth Scale for soil classification (Table 1) [19].

Table I
Sanding grading of sandy soil.

Tyler	Opening (mm)	Classification
-5+9	4.00 to 2.00	Grain
-9+16	1.68 to 1.00	Very thick sand
-16+32	0.84 to 0.50	Coarse sand
-32+60	0.42 to 0.250	Average sand
-60+115	0.21 to 0,125	Thin sand
-115+250	0.105 to 0.062	Very fine sand

Determination of fungi and total bacteria in soil

Under aseptic conditions, 25 g of soil and blender homogenate were weighed, with 225 mL of 0.1% peptone water.

From the initial dilution (10-1), serial dilutions up to 10-5 were prepared. For enumeration of fungi, 0.1 mL of BDD agar (DRBC) agar was inoculated in triplicate, followed by incubation at 25 °C for 5 d. For determination of total bacteria, in triplicate, 0.1 mL of Standard Agar (PCA) agar sample was inoculated, followed by incubation at 35 ºC for 48 h. All materials and media were previously sterilized at 121 °C for 15 min [20].

I. Preparation of the biorremediation test

Homogenization

In a 3000 g vial was added 1868.75 g of non-sterile sandy soil and 625 g of dry sandy soil at 105 ° C (AOAC, 2000). Soil moisture for the bioremediation process was determined according to AOAC (2000) and corrected to 11% weekly with sterile distilled water. The soil was contaminated with 0.25% (m m^{-1}) of the pollutant (fuel oil or petroleum), according to EPA (2001). After soil contamination, the samples were submitted to homogenization (TE - 33I EI - Tecnal rotary agitator, Brazil) for 2 h at 30 rpm. The biosurfactants were added according to the previously determined emulsifying activity. The volume of biosurfactant used in each test was determined according to Equations (2) and (3).

$$m_{\text{Emulsified sunflower oil}} = m_{\text{Total sunflower oil}} \times EA \quad (2)$$

$$VB_{\text{biorremediation}} = \frac{m_{\text{poisoning}} \times VB_{\text{emulsifying activity}}}{m_{\text{emulsified sunflower oil}}} \quad (3)$$

Where: m = mass (g); AE = emulsifying activity (%); $VB_{biorremediation}$ = volume of biosurfactant used in the contaminant bioremediation (mL); $VB_{Emulsifying\ activity}$ = volume of biosurfactant used in the determination of the emulsifying activity (mL).

Bioremediation

In the bioremediation process 5 glass bioreactors were used, of which 1 without the use of a contaminant to determine humidity, with dimensions of 250 mm x 250 mm x 250 mm. In each bioreactor were added 2500 g of sample from the homogenization. The tests were carried out in the external part of the Laboratory of Biochemical Engineering of FURG (latitude 32 ° 04'21.2 "S and longitude 52 ° 10'04.6" W), in open environment exposed to natural environmental conditions, both temperature and humidity and Variations.

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The bioremediation occurred for 90 days. Sampling was performed on days 0, 3, 6, 15, 30, 45, 60 and 90 for analysis of the hydrocarbons. The collected points were selected randomly. The samples were frozen at -80 ° C (New Brunswick Scientific, Model U 535-86, England) and dehydrated by lyophilization (Lyophilizer Model 64132 - Labconco, USA), followed by storage at -18 ° C and subsequently analyzed for hydrocarbon concentration Polycyclic aromatic compounds.

J. Analytical determinations for biorremediation

Determination of pH and temperature

At each collection, the soil and ambient temperature were recorded. The determination of pH in the soil was carried out by a combined bayonet type glass electrode with an express reading at ± 0,01 scale and the soil pH was monitored using a portable digital pH meter (Model Mettler AG 8603 - Toledo, Germany). The soil temperature was measured with a mercury thermometer, with a scale of 0.1. The ambient temperature was determined with maximum and minimum mercury thermometer (Icoterm).

Determination of hydrocarbons

Samples for determination of the hydrocarbons were lyophilized (Lyophilizer Model 64132 - Labconco, USA) and milled in grams. Afterwards, they were conditioned in foil previously calcined in muffle at 450 ºC. The samples were then sent to the FURG's Laboratory of Organic and Metallic Compounds, where polycyclic aromatic hydrocarbons (HAP) were determined in a gas chromatograph (GC-EI-MS QP2010 Plus, Shimadzu, Japan), with a capillary column (Restek, Bellefonte, USA), coupled to a mass spectrometric detector with quadrupole filter, according to the method described by Escarrone et al. [21]. The determined HAPs were: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo (a) -anthracene and chrysene.

III. Results And Discussion

Different surface tension and emulsifying activity data for the biosurfactants produced by Corynebacterium aquaticum and *Bacillus subtilis* CCT 7678 were obtained. According to Batista et al. [22] criteria for the selection of biosurfactants include the reduction of surface tension below 40 mN m^{-1} and the ability to maintain at least 50% of the original emulsion volume 24 h after its formation.

From the results obtained it was possible to verify that both Corynebacterium aquaticum and *Bacillus subtilis* CCT 7678 presented biosurfactants that meet the quality criteria, in relation to the reduction of surface tension (<40 mN m^{-1}) and emulsifying activity (> 50% of the original emulsion volume after 24 h) [22].

Surfactin produced by *Bacillus* spp. Is known as one of the most effective biosurfactants, but it is observed that the best results of surface tension (30.4 mN m^{-1}) and emulsifying activity (99.7%) were obtained in the present work with biosurfactants of Corynebacterium aquaticum. Crosman, Pinchuk and Cooper [10] used Corynebacterium alkanolyticum ATCC 21511 for the production of biosurfactant and this, characterized as phospholipid, was able to reduce the surface tension to 32 mN m^{-1}. Pinto [23] reports that the biosurfactants produced by Corynebacterium aquaticum reduced the surface tension to 31.3 mN m^{-1} using glucose as a carbon source.

The application of biosurfactants was carried out from November/2015 to February/2016. With the sieving of the soil, it was verified that 70.6% by mass was retained in a sieve of 0.250 mm, this soil being classified as medium sand (Table 1).

The soil contains a number of microorganisms capable of metabolizing petroleum compounds. Some authors suggest that the strategy used by anaerobic microorganisms to break aromatic rings is the reductive hydrogenation of the ring, in which the aromatic compound is first transformed into a central intermediate (such as benzoyl-CoA), then the ring is reduced and And finally cleaved by hydrolysis [24].

In the aerobic degradation pathway, molecular oxygen is essential to catalyze the initial hydroxylation of PAH by aerobic microorganisms. The main mechanism for the initial oxidative attack on PAH is through the incorporation of both atoms of an oxygen molecule by dioxygenase, resulting in the formation of cis-dihydrodiols. The dioxygenase that catalyzes these initial reactions consists of a complex enzyme system consisting of a flavoprotein, a ferrodoxin, and a ferro-sulfur protein. The second step in the bacterial oxidation of PAH is the re-aromatization of cis-dihydrodiol through a dehydrogenase to form a dihydroxylated intermediate (catechol). Dihydroxylation of the benzene nucleus is a prerequisite for cleavage of the aromatic ring. The enzymatic fission of the aromatic ring is also catalyzed by dioxygenase. For cleavage to occur, both hydroxyl groups must be placed either in the ortho position or in the meta position.

In the last phase of PAH catabolism, the product resulting from the ring opening is converted into intermediates of the central metabolism (such as acetyl-CoA, succinate and pyruvate) [25].

The pH and temperature variation during the bioremediation process was similar in all experiments, remaining slightly acidic, ranging from 5.7 to 4.9.

According to Zilio et al. [26] the biosurfactant increases the contact surface of the contaminant and consequently facilitates the action of microorganisms. Furthermore, Aislabie et al. [27] reported that organic acids (-COOH) can accumulate during biodegradation, forming cis, cis-muconic acid and 2-hydroximuconic semialdehyde, and the reduction of the pH value may indicate high microbial activity in the treated soil.

The temperature affects the biodegradation of hydrocarbons by their effect on the physical nature and chemical composition of petroleum and its derivatives and on the metabolic rate and composition of the microbial community [28]. The temperature has a significant effect on the rates of hydrocarbon degradation, due to its influence on physical factors like the viscosity of the oil. Also, the viscosity is lower at higher temperatures, thereby increasing the possibility of emulsification, as well as the surface area available for microbial activity and solubilization of petroleum derived compounds. It was observed that the soil temperature remained around 23 ° C and the environment varied from 20 to 26 ° C. The decrease in temperature reduces the microbial activity, mainly by weakening the bonds between the proteins, which compromises the fluidity of the cell membrane and, therefore, the entrance of nutrients and the exit of the products of the metabolism. On the other hand, the increase in temperature provokes the denaturation of the cellular proteins (enzymes), making metabolic reactions unfeasible [29,30].

The biodegradation added to the use of the biosurfactants of the two bacteria acted similarly in the bioremediation of soil contaminated with petroleum with naphthalene, acenaphthene and fluorene being the compounds more rapidly biodegradable, in up to 15 d of bioremediation. Chrysogen was the only PAH that remained during the 90 d of bioremediation, ranging from 56 to 45 µg kg^{-1} with Bacillus subtilis CCT 7678, which corresponds to a degradation of 20% in relation to its initial concentration, with Corynebacterium aquaticum the variation was of 68 to 27 µg kg^{-1}, corresponding to a degradation of 60.3% over the initial, showing that the biosurfactants produced by Corynebacterium aquaticum provided a greater availability of the contaminant for the degradation by the native biota.

According to Leahy and Colwell [31] hydrocarbons differ in susceptibility to microorganism attack. The rates of hydrocarbon biodegradation have been shown to be higher for the saturated ones, followed by the aromatics of low molar mass and aromatics with high molar mass. In the case of PAH, generally the increase in the number of fused rings increases the chemical stability and hydrophobicity of the molecule, making it less susceptible to biodegradation [32]. Manoli and Samara [33] reported that naphthalene, acenaphthene and fluorene have 2 benzene rings and molar mass 128,17, 154,21 and 166,22 g mol^{-1}, respectively and chrysene, has 4 benzene rings and 228 molar mass, 29 g mol-1. This justifies the degradation results obtained in this study.

It was noticed that most PAHs were degraded by up to 45 d of bioremediation of soil contaminated with fuel oil. Only pyrene and chrysene remained during the 90 d assay. The pyrene variation was 162 to 127 µg kg-1 with Bacillus subtilis CCT 7678, corresponding to a degradation of 21.6% over its initial concentration. With Corynebacterium aquaticum, the pyrene variation was 137 to 56 µg kg^{-1}, corresponding to a degradation of 59.2% over the initial concentration. For the chrysogen, the variation was 187 to 104 µg kg^{-1} with *Bacillus subtilis* CCT 7678, the percentage of degradation being 44.4% in relation to the initial amount, already with Corynebacterium aquaticum, the variation was 178 to 67 µg Kg^{-1}, with degradation of 62.4%, relative to the initial concentration. As in the soil contaminated with oil, the biosurfactants of Corynebacterium aquaticum showed higher rates of degradation in soil contaminated with fuel oil in relation to the biosurfactants produced by *Bacillus subtilis* CCT 7678. Among the evaluated contaminants, pyrene and chrysene are the compounds with the highest Number of benzene rings and molar mass, these being 202.25 and 228.29 g mol^{-1}, respectively. According to Chosson et al. [34], the concentration and size of the hydrocarbon molecule influence the susceptibility of petroleum components to biodegradation. Moreover, the higher number of rings increases the particle sorption capacity, leading to lower biodegradability and higher accumulation potential [35].

We observed that benzo (a) -anthracene exhibited oscillations during the bioremediation period with the use of biosurfactants from Bacillus subtilis CCT 7678. Moran et al. [36] reported that some hydrocarbons may be sorbed in the soil matrix, preventing the microbial attack, due to the low availability of contaminant.

It could be observed that the biosurfactants of *Bacillus subtilis* CCT 7678 presented lower reduction of the surface tension and emulsifying activity, compared to the biosurfactants of Corynebacterium aquaticum, which made that the chrysene and anthracene were less available for the native biota to degrade. The decrease of this tension increases the contact area and the breakdown of the compounds in micelle form, reducing the area of contact between the hydrocarbon chains of the surfactant and the water, facilitating the dispersion of the contaminants in the environment and its consequent metabolization by micro - organisms present in the medium [37]

In xenobiotic metabolism, cytochrome P450 is indicated by Berrie et al. [38], as responsible for hydroxylation reactions in bacteria and fungi. In the aerobic degradation of PAH, hydroxylation occurs, evidencing this enzyme in the breaking of the benzene ring, releasing compounds of lower molar mass. Therefore, the lower the chain, the more available the compound will be for microbial degradation. Moreover, it is possible to verify that the break occurs preferably in the ortho and meta position of the benzene ring, which justifies the decrease of the compounds of lower molar mass in decreasing order, as verified in the present study. Thus the biosurfactant promoted the entry of the contaminants into the cells of the microorganisms of the native biota facilitating the action of nonspecific OX-reductase enzymes and cytochrome P 450, as demonstrated by the decrease in their levels.

The degradation of PAHs for soils contaminated with petroleum and fuel oil with the addition of biosurfactants extracted from the bacteria Corynebacterium aquaticum and Bacillus subtilis CCT 7678. It was observed that in the soil contaminated with petroleum, there was a higher rate of Degradation, compared to soil contaminated with fuel oil, so that with the biosurfactants extracted from Corynebacterium aquaticum and Bacillus subtilis CCT 7678, 97.3% and 96.0% of the PAHs present in the oil were degraded, respectively. In the soil contaminated with fuel oil, 93.5% of PAHs were degraded with the use of biosurfactants extracted from Corynebacterium aquaticum, while 89.2% of the compounds were degraded with the use of Bacillus subtilis CCT 7678 biosurfactants.

The biosurfactants of Corynebacterium aquaticum presented greater reduction of surface tension and emulsifying activity, compared to biosurfactants of Bacillus subtilis CCT 7678. A biosurfactant that is able to obtain greater reduction of surface tension and emulsifying activity makes the contaminant more available for the microorganism to degrade, which explains the greater efficiency in the degradation rate of PAH of Corynebacterium aquaticum.

In the present study, the biosurfactants of Corynebacterium aquaticum provided 97.3% degradation of PAH present in the soil contaminated with petroleum and 93.5% of PAH degradation when using fuel oil. Zilio et al. [39] evaluated ex situ bioremediation in Landfarming clay soil using biosurfactants produced by Corynebacterium aquaticum.

The authors verified that 93.8% of the HAP compounds were degraded in 90 d of bioremediation. The results obtained in this study were similar to those obtained by Zilio et al. [30], with the biosurfactants produced by the same bacterium.

IV. Conclusion

Both bacteria were able to produce biosurfactants with promising characteristics to make PAHs more accessible to microbial metabolism, with the best results of surface tension (30.4 mN m^{-1}) and emulsifying activity (99.7%) obtained with *Corynebacterium aquaticum*. The biosurfactants produced by *Corynebacterium aquaticum* were more effective in making the contaminant available for degradation by native biota, both in soil contaminated with petroleum (97.3%) and in fuel oil (93.5%), in relation to the biosurfactants produced by *Bacillus subtilis* CCT 7678, where the degradation was 96.0 and 89.2%, respectively.

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