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Improved Biosurfactant Production by *Bacillus subtilis* SPB1 Mutant Obtained by Random Mutagenesis and Its Application in Enhanced Oil Recovery in a Sand System

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Biosurfactants or microbial surfactants are surface-active biomolecules that are produced by a variety of microorganisms. Biodegradability and low toxicity have led to the intensification of scientific studies on a wide range of industrial applications for biosurfactants in the field of environmental bioremediation as well as the petroleum industry and enhanced oil recovery. However, the major issues in biosurfactant production are high production cost and low yield. Improving the bioindustrial production processes relies on many strategies, such as the use of cheap raw materials, the optimization of medium-culture conditions, and selecting hyperproducing strains. The present work aims to obtain a mutant with higher biosurfactant production through applying mutagenesis on *Bacillus subtilis* SPB1 using a combination of UV irradiation and nitrous acid treatment. Following mutagenesis and screening on blood agar and subsequent formation of halos, the mutated strains were examined for emulsifying activity of their culture broth. A mutant designated *B. subtilis* M2 was selected as it produced biosurfactant at twice higher concentration than the parent strain. The potential of this biosurfactant for industrial uses was shown by studying its stability to environmental stresses such as pH and temperature and its applicability in the oil recovery process. It was practically stable at high temperature and at a wide range of pH, and it recovered above 90% of motor oil adsorbed to a sand sample.

Keywords: Random mutagenesis, biosurfactant, *Bacillus subtilis*, fermentation, oil recovery

Introduction

Biosurfactants are a heterogeneous group of surface-active molecules [1] that contain both hydrophilic and hydrophobic moieties [2]. They are produced on microbial cell surfaces or excreted extracellularly [1, 2]. Microorganisms grown on water-immiscible hydrocarbons produce the majority of known biosurfactants, but some have been synthetized on water-soluble substrates such as glucose, glycerol, and ethanol [1, 3]. These compounds form a diverse group of versatile molecules and are found in a variety of chemical structures such as lipopeptides, glycolipids, fatty acids, neutral lipids, polymerics, and particulate structures [1, 3–6]. Among the many classes of biosurfactants, lipopeptides, usually produced by *Bacillus* strains [6], are specifically interesting because of their remarkable surface properties [4]. In fact, they dramatically reduce surface tension (from 72 to 30 mN/m) [1, 2, 4–6] and have low critical micelle concentrations [2, 5]. These properties create a microemulsion in which micelle formations occur, and hydrocarbons can solubilize in water and vice versa [1, 5]. Worldwide interest in biosurfactants has increased immensely owing to their ability to meet most of the requirements and capabilities of synthetic surfactants [2]. Research in

biosurfactant uses has expanded in recent years in different areas such as pharmaceutical, agriculture, food, and the paper and pulp industry [3]. Moreover, being considered as powerful surfactants, these bioproducts can be deployed by the petroleum industry in microbial enhanced oil recovery (MEOR) processes and bioremediation. Among the numerous potential applications of surfactin, its use in MEOR is considered as one of the most promising methods to recover substantial quantities of the residual oil entrapped in mature oil fields [7, 8]. As more concern has been paid to the protection of the environment, the development of this line of research has become extremely important [3]. In fact, biosurfactants offer the advantages of little or no environmental impact [2]. Therefore, the replacement of conventional chemical surfactants by these biocompounds appears to be a good and efficient approach [1]. Despite their interesting properties, the industrial importance of biosurfactants is dependent upon their ease of production [2]. These compounds cannot compete economically against synthetic surfactants [3] as their low yield is a major factor jeopardizing their popularity.

Recently, efforts have been made to increase yields by focusing on nutritional and environmental factors [2]. Moreover, the bioindustrial production process is often dependent on the use of hyperproducing microbial strains. As previously reported, the production process, even with cheap raw materials, optimized medium and culture conditions, and efficient recovery processes, cannot be commercially viable and profitable until the yield of the final product by the producer organisms is considered naturally high [9]. Indeed, the improvement of microbial strains offers a great opportunity to reduce production costs without significant capital outlay [10], as the enhanced strains, using the same amount of raw materials, are able to synthesize a higher amount of the desired product [11]. Mukherjee *et al*. [9] described that since the capacity to produce a metabolite is bestowed by organism genes, the producer organism genetics turns out to be an important factor affecting the yield of all biotechnological products. Mutation is considered as one of the techniques by which the gene sequence of the bacterial strain under study can be altered in order to induce its activity. Conventionally, among the tools used to enhance the capabilities of biocontrol agents and/or antifungal metabolite producers, we can mention random mutagenesis [4]. The mutant varieties can be produced using various agents; for example, transposons [9], chemical mutagens [12] such as methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) [9], radiation [9, 12], or by selection based on resistance to ionic detergents such as CTAB [9]. Most mutagenic agents might cause damage to the DNA through breakage or base deletion, addition, transversion, or substitution. Consequently, random mutagenesis could affect numerous pathways of the cell machinery [2, 9], which leads to a genetic instability, and thus a potentially improved strain [9, 11–13].

The purpose of the present work was to provide an economical methodology to enhance the biosurfactant production of *B. subtilis* strain SPB1 by using a combination of nitrous acid and ultraviolet rays to induce mutation. This investigation aimed to develop a cost-effective and simple approach in order to increase the biosurfactant productivity. This work also describes the biosurfactant properties of the selected mutant under extreme environmental conditions, its biological activity, and its potential for industrial applications.

Materials and Methods

Bacteria and Culture Conditions

The biosurfactant-producing bacterium was obtained from Tunisian soil and isolated in our laboratory. It was identified as *B. subtilis* SPB1 (HQ392822) and characterized as a producer of a lipopeptide biosurfactant with high hemolytic and emulsification activities [14]. As described by Mnif *et al*. [15], the mass spectroscopic analysis of the culture filtrates of SPB1 indicated the presence of surfactin, iturin, and fengycin isoforms. In this work, we used this strain as a reference.

The *B. subtilis* SPB1 biosurfactant production was performed as reported by Ghribi and Chaabouni [16], with slight modifications. At time-defined intervals, samples were collected and submitted to analysis in order to determine biomass concentration and biosurfactants production. All experiments were performed in duplicates.

Induction of Mutagenesis

Treatment of vegetative cells with nitrous acid. A single colony of the wild-type strain *B. subtilis* SPB1 was dispensed into 3 ml of LB medium and then incubated overnight at 150 rpm and 37°C. A 0.5 ml sample of this culture was diluted into 50 ml of fresh LB medium and incubated on a shaker (Lab Companion SI-300R) until an optical density (OD_{600nm}) of 3.5 was reached. This culture was diluted with LB medium to OD_{600nm} of 0.1. Subsequently, a fresh nitrous acid solution (10 mg/l) and phosphate buffer (0.1 M, pH 7; 744 pH meter; Metrohm, Switzerland) were added to the centrifuged cells. The solution was totally homogenized and then incubated at 150 rpm and 37°C, for a variable period of time (15, 30, 45, and 60 min) [11]. Serial dilutions of both treated and non-treated cells were moved to nutrient agar and incubated overnight at 37°C, and colony forming units (CFU) were counted. This was carried out in three replicates for each CFU determination.

Percentages of lethality were measured as follows: 100 × [1 – (CFU of treated suspension/CFU of non-treated suspension)].

Treatment of vegetative cells with UV rays. Mutagenesis with short wavelength ultraviolet irradiation was carried out using a Philips tube (15 W). The cell suspension (OD_{600nm} of 0.1) was distributed in Petri plates (10–15 ml in each plate) and exposed to UV radiation for variable periods (10, 20, 30, 40 min) while keeping the distance of the UV source fixed at 20 cm [13]. Serial dilutions were used to calculate the lethality percentage as described above.

Screening and Isolation of Potential Mutant Strains

Hemolysis activity. Each strain was spotted onto blood agar plates and incubated for 24 h at 37°C. The appearance of a clear zone around the colonies is an index of biosurfactant production [17]. The parent strain *B. subtilis* was used as a control in each plate, and mutants overproducing biosurfactant were selected based on their high hemolysis activities, since the size of the developed halos is in proportion to the amount of the produced biosurfactant [18].

The improvement of biosurfactant production was calculated as follows:

$$\text{Improvement rate (\%)} = ((Hm/Cm) - (Hwt/Cwt))/(Hwt/Cwt)$$

where Hwt and Hm are respectively the hemolysis zone surrounding the wild type and the mutant strain (mm), and Cwt and Cm are respectively the colony size of the wild type and the mutant (mm).

Emulsification activity. Each mutant was evaluated for its ability to produce biosurfactant. A screw-capped tube containing 0.5 ml of cell-free culture broth, 7.5 ml of Tris-Mg (20 mM Tris HCl (pH 7.0) and 10 mM $MgSO_4$), and 0.1 ml of kerosene were prepared. After a vigorous agitation with a vortex (SCI LOGEX MX-S), the tubes were permitted to rest for 1 h. Emulsification activity (EA) was defined as the optical density at 540 nm [19] measured via a spectrophotometer (UV-1800; SHIMADZU, Japan). Assays were carried out in triplicates.

Effects of Temperature and pH on Biosurfactant Stability of Wild-Type and Mutant Cells

The effect of pH on the biosurfactant stability was determined by adding to the free cell culture broth the following buffers (all at a concentration of 0.1 M): glycine HCl (pH values 2 and 3), sodium acetate (pH values 4 and 5), sodium phosphate (pH values 6 and 7), Tris-HCl (pH values 8 and 9), and glycine NaOH (pH 10). The mixtures were incubated at ambient temperature for 1 h and the stability of the biosurfactant was explored [1].

The thermostability of the produced biosurfactant was studied by determining the residual activity of the culture broth supernatant after a heat treatment at diffrent temperatures (25°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C) for 60 min. Activities were determined at 37°C [1].

Application of Biosurfactant in Motor Oil Removal from Contaminated Sand

The sand sample used in this study was collected from Tunisian Djerba beach. It was sifted to remove impurities and large particles and incubated overnight at 105°C to remove humidity [20]. As described by Luna *et al*. [21], the suitability of the biosurfactant for enhanced oil recovery was determined using 60 g of beach sand impregnated with 5.0 ml of motor oil. Then, 20 ml of the cell-free broth was transferred to 250 ml Erlenmeyer flasks that contained fractions of 10 g of contaminated sand. The samples were incubated for 24 h on a rotary shaker (150 rpm) at 27°C and centrifuged (HEHICH Zentrifugen Rotina 380 R, Germany) at 5,000 ×*g* for 10 min in order to separate the laundering solution and sand. The aqueous phase was eliminated carefully, and the sand was rinsed with distilled water to remove the non-adsorbed oil and to take off the remaining surfactant solution from the sand to prevent therefore the possible formation of an emulsion with the extracting solvent later when determining the remaining oil in the sand [20].

The quantity of the adsorbed oil was measured after its extraction by an organic solvent (hexane) [22, 23].

Phytotoxicity Assay

A static test based on seed germination and root elongation of the vegetable *Raphanus sativus* was used to evaluate the phytotoxicity of the biosurfactant, following the methods described by Tiquia *et al*. [24]. Sterilized Petri dishes containing Whatman No. 1 filter paper were used to determine the toxicity. A solution at concentration of 1 g/l of the crude biosurfactant was prepared with distilled water and used to inoculate 10 seeds.

After 5 days of incubation at 27°C in the dark, seed germination, root elongation (≥5 mm), and the germination index were determined as follows:

Relative seed germination (%) = (number of seeds germinated in the extract/number of seeds germinated in the control) × 100.

Relative root length (%) = (mean root length in the extract/mean root length in the control) × 100.

Germination index = [(% of seed germination) × (% of root growth)]/100%.

Results and Discussion

Effects of Nitrous Acid and UV Rays on Cell Viability

The *B. subtilis* strain was subjected to random mutagenesis by a combination of nitrous acid treatment and UV irradiation. According to the literature, nitrous acid (HNO_2), derived from sodium nitrate ($NaNO_3$), acts as a chemical mutagen by deamination of the NH_2 group of adenine and cytosine to another group, thus corrupting the base pairing [25]. In addition, the penetration of UV rays through the

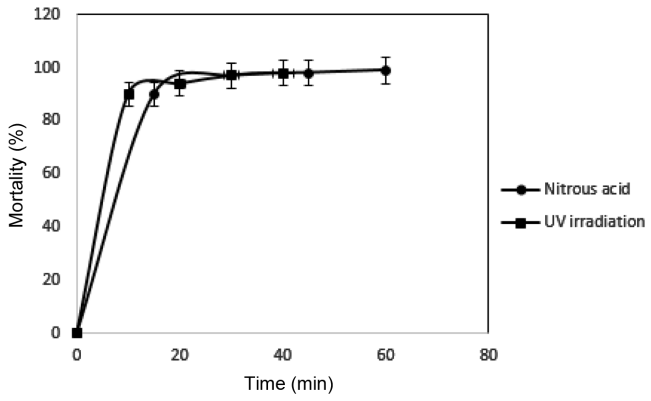

Fig. 1. Effects of nitrous acid and UV rays on cell viability.

cell membrane can cause a loss in its segment, makes the cross-linked DNA unopened, and prevents replication and transcription of the DNA. As a result, this DNA damage can change the biological activity, alter protein structure, and cause bacterial mutation even after death [26–28]. Hence, the UV irradiation stands as the most widely used physical mutagen [26].

The lethal doses of the selected mutants are presented in Fig. 1. It is clear that vegetative cells of the SPB1 strain were sensitive to UV and nitrous acid, which is in accordance with the results found by Ghribi *et al*. [11], who proved the sensitivity of *Bacillus thuringiensis* to these mutagenic agents. As shown (Fig. 1), after 15 min of incubation in fresh nitrous acid, less than 10% of survivors were detected. In addition, after 10 min of UV exposure, 90% of the initial cells had died. The rate of dead cells reached 98% after 40 min of exposure to UV light. As previously proved, the amount and time of exposure of the mutagen can cause high bacteria lethality (about 90–99%) [26, 27].

As remarked, the two stages of mutagenesis led to a survival rate less than 10%. Our results correspond, firstly, to those of Ghribi *et al*. [11] and Vahed *et al*. [13], who treated *B. pumilus* SG2 and *B. thuringiensis* with nitrous acid and UV irradiation, respectively, and, secondly, to Tahzibi *et al*. [2], who used NTG as mutagenic agents to treat *Pseudomonas aeruginosa*. It is believed that an intermediate dose could allow the survival of 10% of the treated cell population and generates more mutants with improved titer [29].

Effects of Nitrous Acid and UV Rays on Cell Morphology

The colony morphology of the isolated mutants was circular and opaque, and no unusual phenotypes were observed. Moreover, we did not observe any differences with the wild strain morphology under the optical microscope. This observation was in accordance with what was observed by Azzouz *et al*. [30] and Ghribi *et al*. [11] when they treated *B. thuringiensis* under the same conditions. However, Kim *et al*. [31] reported that the mutants derived from *B. thuringiensis* var. *israelensis* differed from the wild strain after their treatment with MNNG.

Selection of Mutants

Isolation of mutants, overproducing biosurfactant, with the blood agar plate test has been previously reported [32, 33]. In this study, we tested all the survivors for their hemolytic activity. As described by Beggs [34], the fewer the positive mutant cells, the more negative the mutant strains are in the survival cells. As shown in Table 1, among 327 colonies of the survivor isolated from nitrous acid treatment, only 36% of cells presented clear zones with larger diameter, and all other mutants showed either lower or similar hemolytic activities when compared with the wild-type strain. In fact, mutations would affect several metabolic pathways related to the regulation of bioproducts synthesis, which explains the fluctuation in the improved biosurfactant production from one mutant to another [30].

Among the isolated overproducer mutants, only one mutant, named M35, was selected on the basis of the

Table 1. Summary of results of nitrous acid and UV irradiation mutagenesis.

Round	1st	2nd	2nd	3rd
Treatment	NA	NA	UV	NA
Treated strains	SPB1	M35	M35	M35-5
Total number of screened survivors	327	11	44	14
Total number of biosurfactant overproducer strains	119	4	20	10
Total number of cells with improved biosurfactant production of more than 20%	3	2	2	1
Selected mutants	M35	M35-5	M35-43	M2
Hemolytic activity improvement (%)	31	63	59	109
Emulsifing activity improvement (%)	33	60	52	118

balance of hemolytic activity improvement of 31%.

To isolate more interesting overproducing mutants with high biosurfactant production improvements, a second round of mutagenesis by UV and nitrous acid was applied to *B. subtilis* M35. It seems from this second round of mutagenesis that from the total survivors, 18% and 5% of cells obtained, respectively, from nitrous acid and UV treatments were improved regarding biosurfactant production, with a rate above 20%. This indicated that nitrous acid may be more efficient than UV in generating *Bacillus* mutants with improved biosurfactant production of >20%. However, previous work showed the benefit of using UV radiation compared with nitrous acid treatment in leading to *Bt* mutants with improved delta-endotoxin production of >30% [11].

Among the preselected survivors obtained from the treatment of *B. subtilis* M35 with nitrous acid and UV, mutants M35-5 and M35-43 were respectively improved regarding biosurfactant production. Improvements of biosurfactant production by M35-43, compared with M35 and SPB1, was, 28% and 59%, respectively. However, it was 31% and 63% for the mutant M35-5 compared with M35 and SPB1. These high rates of improvement resulted in *B. subtilis* M35-5 being selected for a third round of mutagenesis by nitrous acid.

As performed for the wild strain SPB1, we conducted a similar experimental approach for the third time on the selected mutant M35-5. Just one mutant, designated *B. subtilis* M2, was improved concerning biosurfactant production (109%), compared with the wild strain SPB1. Therefore, a lower sensitivity of *B. subtilis* cells to nitrous acid was observed when passing from the second to the third round of mutagenesis, as only 7% of survivor cells were showing an improved biosurfactant production higher than 20% for the third round of mutagenesis compared with 18% for the second one. This indicated that nitrous acid may lose efficiency in generating *B. subtilis* mutants with improved biosurfactant production of >20% from one round to another. This is in accordance with the results found by Ghribi *et al*. [11], who described that second mutations might nullify the positive impact of first ones, thereby decreasing the frequency of obtaining improved mutants via second mutagenesis.

As described by Pag *et al*. [35], the hydrophilic part of a biosurfactant is proposed to initiate electrostatic interaction with the membrane components of microbes. On the other hand, the hydrophobic part is supposed to allow the peptides to be inserted through the membrane. As biosurfactants would cause lysis of erythrocytes, an association between surfactant production and hemolytic activity was deduced [36]. Therefore, the blood agar test has been used for the isolation of lipopeptide biosurfactants [33, 37]. Many researchers [4, 32, 34, 38, 39, 40] recommended the use of blood agar lysis as a simple, easy, rapid, and primary screening method of biosurfactant-producing strains, in which samples with positive results are later subjected to biosurfactant-activity tests in liquid media.

However, it is not clear whether blood agar lysis should be used to screen for biosurfactant production since the extent of hemolytic zone formation on blood agar plates is not necessary due to the presence of biosurfactant and may be caused by different compounds, such as divalent ions, virulence factors, and other hemolysins produced by the microbe under investigation [2, 37]. Some researchers suggested that this technique should be propped by other methods based on surface activity measurements [32, 38]. The most widely used methods for the measurement of surface activity are based on emulsification tests [41]. Moreover, Toren *et al.* [42] noticed a good correlation between the emulsification activity and the quantity of biosurfactant. Thus, in the next stage, we analyzed the isolated strains for their emulsification activity. Compared with the wild-type strain, mutants with higher emulsification activity were selected. They were then carefully evaluated for their ability to produce biosurfactant, with the wild-type strain being used as a control.

In this work, we noted a good correlation between the blood agar lysis and the emulsification activity (Table 1). The isolates, which were positive in blood agar lysis, also gave a high emulsification property. The values of improved emulsification activity ranged from 36% to 118% for mutants M35, M35-5, and M2.

In conclusion, after three rounds of mutagenesis by nitrous acid applied respectively on the *B. subtilis* wild-type (wt) strain, the mutant M35, and finally the mutant M35-5, a mutant named *B. subtilis* M2 showed the highest hemolytic and emulsification improvement (109% and 118%, respectively). Therefore, it was selected for all further experiments, with more than 2-fold biosurfactant production.

Many previous reports showed the overproduction of biosurfactants, using random mutagenesis. Indeed, Gong *et al.* [43] described that, after treatment with ion beam, a high-producing surfactin mutant was obtained from *B. subtilis* E8. A mutant with a more than 1.5-fold biosurfactant production was obtained by gamma irradiation in *Pseudomonas aeruginosa* MR01 [44]. Moreover, a mutant of a recombinant strain (*B. subtilis* MI 113), induced by MNNG, produced surfactin in the range of 8–50 g/l when grown on flour

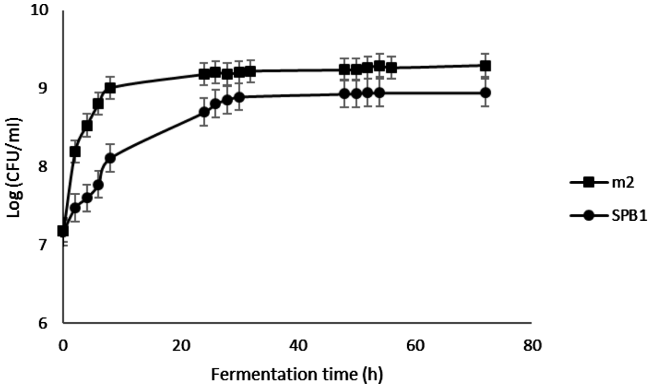

Fig. 2. Kinetics of growth of *Bacillus subtilis* wild type strain (●) and the mutant M2 (■).

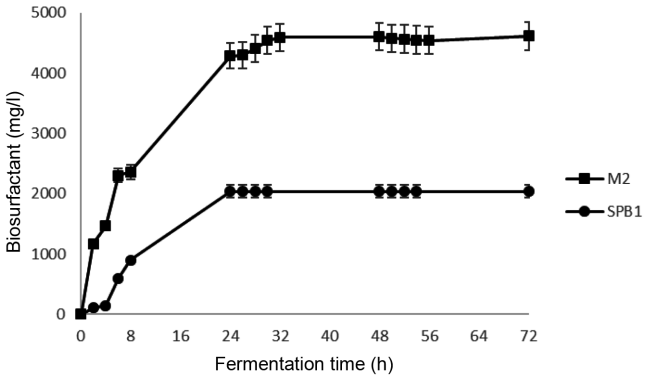

Fig. 3. Kinetics of the biosurfactant production by *Bacillus subtilis* wild-type strain (●) and the mutant M2 (■).

from soybean or its extract [45].

Comparison between SPB1 Biosurfactant and *Bacillus subtilis* M2 Biosurfactant

Microbial growth and biosurfactant production. The kinetics of growth of both the wt and the mutant strains are given in Fig. 2.

Similar to the wt, the biomass concentration of the mutant rose instantly after inoculation, indicating an adequate growing state in the inoculum. After 24 h, it reached its maximum of about 18.5 × 10^8 and 8.9 × 10^8 CFU/ml, respectively, for the mutant and the wt, demonstrating that the culture has reached the stationary phase, as according to what were noted by Afsharmanesh *et al.* [4]. At this growth phase, we obseved a concentration of the biosurfactant biosynthesis (Fig. 2). It started since the first hour of incubation and showed a high level of output in the next 30 h. As they facilitate nutrient transport and host-microbe interactions, and act as biocides, some biosurfactants may play vital roles in the survival of the producing micro-organisms [46]. The maximum of the biosurfactant production obtained under the parent strain was only 2,040 mg/l after 48 h. However, when using the mutant strain, the production was enhanced by more than 2-fold to reach 4,560 mg/l.

By comparison with the wt strain, it is clear that the *Bacillus subtilis* M2 improved biosurfactant yield and productivity from 22.92 mg/10^7 CFU to 24.65 mg/10^7 CFU and from 42.5 mg·l^{-1}·h^{-1} to 95 mg·l^{-1}·h^{-1}, respectively (Table 2).

Biosurfactant stability regarding emulsification activity. An emulsion is formed when one liquid phase is dispersed in another (continuous), as microscopic droplets. Most biosurfactants can emulsify or solubilize various hydrocarbons at different rates. However, their inability to stabilize the microscopic droplets can cause the inadequate emulsification of some hydrocarbons. The activity and stability of biosurfactants can be affected by environmental elements, such as temperature, pH, and salinity, and thus the study of leverage of these factors is important when considering specific applications for such compounds [21].

The thermostability of the acellular culture broth of the wt and *B. subtilis* M2 was tested over a wide range of temperature (25–100°C) for 1 h. The residual activity was determined and is presented in Fig. 4A. As shown, temperature ranging from 25°C to 60°C did not have any significant effect on the stability of the emulsification activity, indicating the thermostability of both biosurfactants. The optimum of emulsification activity was obtained at temperature lower than 40°C. The *B. subtilis* M2 bioemulsifier retained more than 84% and 78% of its activity after incubation during 60 min at 80°C and 90°C, respectively; in contrast, the wt biosurfactant retained only 55% of its activity at these temperatures [16]. Heating the supernatant containing biosurfactant produced by the mutant strain to

Table 2. Biosurfactant and cell production in glucose-based medium by the wild strain SPB1 and *Bacillus subtilis* M2.

Strain	Biosurfactant (mg/l)	CFU (10^8 CFU/ml)	Biosurfactant yields (mg 10^7 CFU)	Biosurfactant productivity (mg·l^{-1}·h^{-1})	Biosurfactant production improvement (%)
Wild strain	2,040	8.9	22.92	42.5	0
Mutant M2	4,560	18.5	24.65	95	123.5

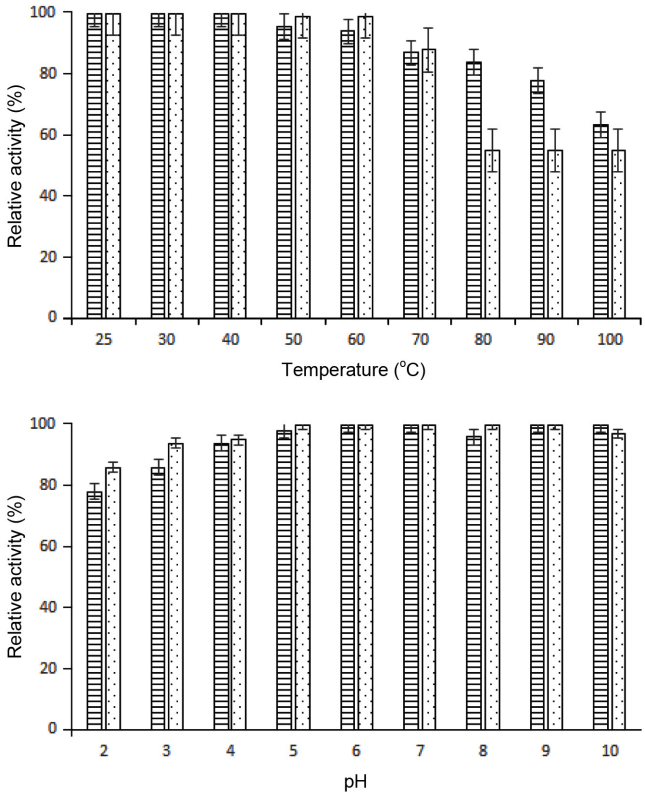

Fig. 4. Effects of temperature (**A**) and pH (**B**) on the activity of the crude biosurfactant produced by *Bacillus subtilis* wild-type strain (□) and the mutant M2 (▤).

100°C caused a decrease in its performance up to 63%, which remained higher than that found by the wt (55%). As described by Varadavenkatesan and Murty [47], when they varied the temperature from 0°C to 121°C, both the surface tension and emulsification index (E_{24}) of the cellular culture broth obtained from *B. siamensis* showed little variation and remained nearly constant. The result was also in accordance with those reported by Ben Ayed *et al*. [48] showing that the biosurfactant from *B. mojavensis* exhibited an emulsification activity over a wide range of temperature from 20°C to 100°C, and it could retain more than 60% of its emulsification activity at 100°C. Similar results were observed in the literature [3, 49]. Moreover, synthetic surfactants such as SDS exhibit a significant loss of emulsification activity starting from 70°C [50].

The effect of pH on surface activity has been reported for biosurfactants from different microorganisms [3]. Fig. 4B shows the effect of pH on the biosurfactant stability. The emulsification activity of both wt and mutant biosurfactants remained relatively stable (80–100%) against pH changes between 4 and 10, showing higher stability at alkaline pH than acidic conditions. At pH equal to the isoelectric point, there is no electrostatic repulsion between neighboring molecules owing to the protonation of carboxylic side chains of glutamic or aspartic acids in the peptide portion, and therefore biosurfactants tend to coalesce and precipitate. This precipitation was associated with the decrease of its activity [40]. In fact, at pH 2 (acidic pH), the EA of the wt and the mutant biosurfactants retained, respectively, only 86% and 78% of their activity. The *B. subtilis* M2 biosurfactant was very stable over a considerable pH. Our results are in accordance with those of Ben Ayed *et al*. [48] showing that a biosurfactant obtained from *B. mojavensis* A21 might be useful in acidic and alkaline environment conditions since it was active over a wide pH range from 2 to 10. Moreover, Varadavenkatesan and Murty [47] reported that variability in the surface tension and emulsification index, when using fermentation broth of *B. siamensis*, was not profoundly observed when involving changes in pH from 2 to 12. In addition, many researchers observed similar results [51, 52].

Utilization of *B. subtilis* Biosurfactant in Removal of Hydrophobic Contaminant Adsorbed to Sand

Sea oil spills may cause environmental accidents, resulting

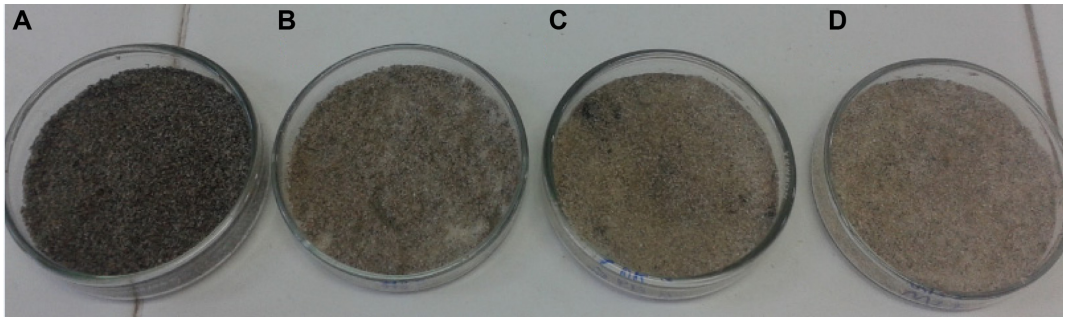

Fig. 5. Oil recovery test: (**A**) untreated sample, (**B**) sand treated by water, (**C**) sand treated by a cellular culture broth of the wild-type strain, (**D**) sand treated by a cellular culture broth of the mutant strain.

in several ecological and social problems [8]. Petroleum hydrocarbon compounds bind to soil components and thus they are difficult to be removed because of their hydrophobicity [53]. Surface-active compounds can present a positive contribution on the remediation process, as they improve the solubility and the removal of hydrophobic compounds from contaminated soils and, therefore, their biodegradation [54]. The majority of crude oil components present low solubility in water and are bound to soil particles, which would reduce the availability of microorganisms for degradation. Biosurfactants are acknowledged for their ability to emulsify hydrocarbons by improving their water solubility, reducing surface tension, and elevating the displacement of oil substances from soil particles. These properties can enhance the degradation of hydrocarbons in the environment, potentially allowing biosurfactants to be useful tools for a better control of oil spill pollution [8, 21]. In order to investigate the application of the biosurfactant produced by *B. subtilis* wt and mutant strains in contaminant removal, we realized a preliminary experiment using the cell-free broth in removing hydrocarbons from sand samples [55].

Satisfactory results were obtained regarding the removal of motor oil adsorbed to sand samples by the cell-free broth (crude biosurfactant) of each strain, with a removal rate of 90% (Fig. 5). As remarked, the use of the biosurfactant without purification steps was possible since the crude biosurfactant can be practically as effective as the isolated biosurfactant in removing oil, and thus would decrease the production costs [55]. Promising results have been obtained by biosurfactants produced from *Bacillus* species. In fact, Pereira *et al*. [7] found that three strains of *B. subtilis* were effective in oil recovery from sand pores, with rates between 19% and 22%. Moreover, 85% of motor oil was detached from the contaminated sands when using the cell free supernatant from *B. subtilis* CN2 [53]. More recently, Zhang *et al*. [56] demonstrated the considerable ability of biosurfactants produced from *Bacillus atrophaeus* KP5-2a to remove 94% of the crude oil adsorbed on sand.

Biosurfactant Phytotoxicity

The germination index (GI), which combines measures of relative root elongation and relative seed germination, was used to evaluate the toxicity of the *Bacillus subtilis* M2 biosurfactant to the vegetable *Raphanus sativus*. We found a GI of 83% for mutant biosurfactant solutions of 1 g/l. It was also possible to visualize the leaves and the elongation of secondary roots for all the conditions tested. As a GI value of 80% has been used as an indicator of the absence of phytotoxicity [24], the results obtained in the present study indicate that the solution tested did not have an inhibitory effect on root elongation or seed germination in the analyzed vegetables.

A series of experiments were conducted by Chandankere *et al*. [57] to assess the level of toxicity of the biosurfactant obtained from *B methylotrophicus* against *T. aestivum*, *R. sativus*, *V. radiate*, and *B. napus*. As a results, 100% germination of the seeds was obtained for the biosurfactant. *V. radiate* (185) had the highest growth index, followed by *T. aestivum* (158), *B. napus* (132), and *R. sativus* (102). Biosurfactant solutions, from *P. aeruginosa*, of 175, 350, 525, and 700 mg/l showed a growth index of 245%, 187%, 132%, and 86%, respectively [55].

In a conclusion, through random mutagenesis, by UV irradiation and nitrous acid treatments, a mutant with the ability to produce biosurfactant in a large amount compared with the wt was selected. The newly produced biosurfactant was effective over a wide range of temperatures and pHs, able to remove hydrophobic contaminants, and presented lower toxicity, thus justifying the possibility of its industrial use, especially in environmental application. Development of nonpathogenic, safe, potent, and high-yielding mutants and recombinant varieties is expected to be achieved in the near future. The focus on developing novel recombinant hyperproducer strains will enhance industrial biosurfactant production and allow their commercialization by making their production process cheaper and safer.

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