Application of DNA Shuffling as a Tool for Hydrolase Activity Improvement of *Pseudomonas* Strain

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Abstract: Biotechnology is considered one of the most influential technologies in various areas of human life, including health, economics, and the environment. Protein engineering is one of the major biotechnology tools in the field of modification and advancement of biocatalysts capabilities. Among the most effective protein engineering methods, in particular, to improve the industrial strain capabilities, is the shuffling genome method. This study aimed to follow knowledge and biocatalysts engineering techniques based on DNA shuffling methods. In the first step, two procedures were followed (DES method and compatibility according to the concentration gradient of Diazinon) to obtain mutant strains. Acquired mutant strains from both methods were resistant to high concentrations of poison up to 3000 mg/L. The activity of these strains also demonstrated their elevated activity compared to parent samples. The highest activity was related to four strains IR1.G1, IR1.D8, IR1.D4, and IR1.D5, which were 0.234 U/ml, 0.1 U/ml, 0.098 U/ml, and 0.066 U/ml, respectively. The improved strain was obtained via the concentration gradient of the diazinon method (IRL1.G1 strain) in comparison with IRL1.D8 strain (owning highest activity through DES method) possesses excessive activity in 3000 mg/L concentration of Diazinon. The evaluated results of first-generation genome shuffling of strains (the first round of protoplast fusion) also indicated that those shuffled strains with the ability to grow in the vicinity of the toxin (3000 mg/L concentration of Diazinon) showed better activity than obtained mutated strains by both methods (concentration gradient of the toxin and the DES method). In the final stage, the best results were related to IRL1.F2, IRL1.F3, and IRL1.F1 shuffled strains with 0.541 mg/L, 0.523 mg/L, and 0.509 mg/L, respectively. The highest activity belonged to the IRL1.F2 genome shuffled strain (first round of protoplast fusion). This strain could grow in a high concentration of toxin, and also, the activity was increased 30, 3.6, and 2.3 times in comparison with the parent strain (IRL1), IRL.D8 mutant, and IRL1.G1, respectively.

Keywords: Diazinon; genome shuffling; improved strain; protein engineering.

1. Introduction

Organophosphorus (Op) insecticides have been used for agricultural purposes globally in recent decades. Numbers of OP compounds can be named, such as Malathion, Dichlorvos, Parathion, and Chlorpyrifos. OPs target Acetylcholinesterase (AChE), which is a vital enzyme in hydrolyzing neurotransmitter acetylcholine, leading to inactivation of AChE and thus

causing toxicity [1-4]. Accordingly, the usage of these compounds generates health concerns toward humans and other living creatures. To conquer this problem, many efforts have been made, and different approaches have been used to eliminate the contamination arising from the use of these compounds. Methods such as physical, chemical, and biological can be mentioned as decontamination strategies [5-9]. Biodegradation is regarded as an outstanding method due to some prominent characteristics like quick action, environment-friendly, safe and reduced toxicity of products [10-14].

Previous studies have proved that some microorganisms can decontaminate specific toxic compounds [15]. Several isolated bacteria, namely *Flavobacterium sp.* and *Pseudomonas diminuta MG* have shown to own significant capabilities to hydrolyze Ops [16, 17]. *Pseudomonas sp.* is considered an outstanding candidate for Ops biodegradation and is used for the decontamination of contaminated effluents and agricultural soils [18, 19].

Despite numerous benefits of using bacterial strains in the biodegradation process, some application aspects of these strains deter their frequent use. One of the limiting factors is substrate availability. The cytoplasmic enzymes of these bacteria are responsible for their bioactivity, which limits access to the substrate. On the other hand, they require a longer time to act on the substrate, thus decreasing their quality of efficiency [20,21].

In case of having access to the details of enzyme properties, researchers have attempted to improve the hydrolase activity of several enzymes through several methods [22]. Genome shuffling is widely used for increasing the production of metabolites by bacterial strains, improving substrate uptake as well as enhancing strain tolerance. This technique combines the advantage of multiparental crossing allowed by DNA shuffling together with the recombination of entire genomes normally associated with conventional breeding, or through protoplast fusion, that increases the recombination process. Stemmer and co-workers first presented the method of genome shuffling in 2002 when it was used to improve the production of tylosin by *Streptomyces fradiae*. Nowadays, it is used in many experiments for increasing the production of metabolites by bacterial strains, improving substrate uptake, and enhancing strain tolerance. Genome shuffling is a major milestone in strain-improvement technology and metabolic engineering [23, 24]. In this study, we made an effort to improve the properties of *Pseudomonas aeruginosa* to degrade Diazinon, using the genome-shuffling technique. Toxicity resistance to Ops and an increase in whole-cell activity is aimed in this study. The main goal is to obtain a mutant strain, which can grow in high concentrations of Diazinon and, on the other hand, degrade Diazinon efficiently in a short time.

2. Materials and Methods

2.1. Bacterial strain.

Wild type *Pseudomonas aeruginosa* JQ917006.1 (IRLM.1) was isolated from industrial wastewaters (effluents of pesticide producing factories and their contaminated soil) in Iran (Genbank accession number: JQ917006.1).

2.2. Chemicals and reagents.

Diazinon (99.5% purity) was purchased from Sigma-Aldrich. The other chemicals and solvents were obtained from Merck, Germany.

2.3. Native strain tolerance test against Diazinon.

In the beginning, the isolated *P. aeruginosa* strain IRLM.1 was cultured in mineral salt medium (MSM) broth (0.1 g/L NaCl, 0.2 g/L KCl, 0.5 g/L $(NH_4)_2SO_4$, 50 mg/L $CaCl_2.H_2O$, 2% (w/v) glucose) without Diazinon and was incubated at $37°C$ until OD_{600} reached to 0.5 (measured by NanoDrop spectrophotometer, Thermo Scientific, DE, USA). The bacterial suspension was centrifuged (at 7000 rpm for 5 min) and then was washed with MSM broth without glucose for several times. In the next step, bacterial crude was added to MSM agar (0.1 g/L NaCl, 0.2 g/L KCl, 0.5 g/L $(NH_4)_2SO_4$, 50 mg/L $CaCl_2.H_2O$, 15 g/L agar) and broth without glucose, supplemented with 1000 to 3000 mg/L diazinon and then incubated for 72h at $37°C$. Genome shuffling was carried out through two routes: 1. Chemical adaptation (IRLM1.G strain) 2. DES mutation inducing material (IRLM1.D strain).

2.4. Creation of mutant library (IRLM1.G) via chemical adaptation.

The first population obtained by chemical adaptation, which included a gradual increase in diazinon amount within 1200 h. This could be explained in this way that after confirming the resistance of native bacteria, they were exposed to higher concentrations of Diazinon (2500-3000 mg/l) on MSM agar. Afterward, the cultures were incubated for 72 h at $37°C$ and were kept in a dark place to avoid photo-degradation of Diazinon.

2.5. Creation of mutant strains (IRLM1.D) by diethyl sulfate (DES).

The native strain was cultured in the MSM medium and was incubated at $37°C$ until the OD_{600} reached 0.5. The media was treated with 0.5%, 1%, 1.5%, 2%, 2.5%, and finally, 3% of DES (Sigma Aldrich) and then was incubated for 72 h at $37°C$. The bacterial suspension was centrifuged (at 7000 rpm for 5 min) and then was washed with PBS. Eventually, the obtained pellet was cultured in LB agar.

2.6. Screening based on growth in the presence of monocrotophos and biodegradation of Diazinon in the liquid medium.

The obtained mutant library was cultured on LB agar and incubated at $37°C$ for 48 h. Then a thin layer of agarose 0.7% containing 50 mM phosphate-citrate buffer and 0.5 mM monocrotophos was added to media and incubated for 1 h at $37°C$. The colonies were selected based on the magnitude of the halo. The selected colonies were inoculated to MSM broth containing Diazinon (2000, 2500, and 3000 mg/L) as the sole carbon source. OD_{246} was measured at 0, 24, 48, and 72 h using NanoDrop (Thermo Scientific, DE, USA). As mentioned earlier, all the experiments related to Diazinon were done in dark condition due to the effects of light on Diazinon [25-29].

2.7. Protoplast preparation and inactivation treatment.

Protoplasts were prepared [30] except for a few modifications. The bacterial cells were cultured on MSM broth overnight at $37°C$, and then cells were harvested by centrifugation at 7000 rpm for 5 min. The bacterial cells were suspended in 10 ml protoplast buffer (PB), containing 10 mM Tris-HCl, 20 mM $CaCl_2$, and 0.5 M sucrose along with 1 mg/ml lysozyme and incubated at $60°C$ for 2 h for inactivation. Protoplast formation was observed microscopically by a light microscope.

2.8. Genome shuffling.

The starting populations for genome shuffling were mutants-library-generated by the gradually high concentration of diazinon adaptation and DES factor. Inactivate protoplasts were mixed in a ratio of 1:1 and collected by centrifugation followed by resuspension in the ratio of 9:1 of PEG 6000 (60%) and PB, respectively, and incubated for 30 min at 37°C. The fused protoplasts were suspended in 5ml PB and were centrifuged at 3000 rpm for 20 min at 25°C. The pellets were regenerated on regeneration medium (20 mM magnesium chloride, 0.5 M sucrose, and 10.5% BSA) for 18 h at 37°C.

2.9. Analytical method.

The screening was performed based on growth in the presence of monocrotophos and fusants were selected. Moreover, diazinon biodegradation and resistance against Diazinon were evaluated according to which previously described.

3. Results and Discussion

3.1. Construction of the initial strains population for genome shuffling.

The first population of the adapted mutant library was achieved by slowly increasing the concentration of Diazinon from 1000 to 3300 mg/L over 72 h. The mutant strains (IRLM1.G) were able to grow in 3250 mg/L of Diazinon, while wild type strain was tolerated for growing in 2900 mg/L. The results are shown in Table 1.

Table 1. First adapted mutant library.

Concentration of diazinon (mg/L)	Sample	Time		
		24 h	48 h	72 h
1000-2600	IRLM1.G	+	+	+
	IRLM1	+	+	+
2700	IRLM1.G	+	+	+
	IRLM1	-	+	+
2800	IRLM1.G	+	+	+
	IRLM1	-	-	+
2900	IRLM1.G	+	+	+
	IRLM1	-	+	+
2950	IRLM1.G	+	+	+
	IRLM1	-	-	-
3000	IRLM1.G	+	+	+
	IRLM1	-	-	-
3050	IRLM1.G	+	+	+
	IRLM1	-	-	-
3100	IRLM1.G	+	+	+
	IRLM1	-	-	-
3150	IRLM1.G	+	+	+
	IRLM1	-	-	-
3200	IRLM1.G	+	+	+
	IRLM1	-	-	-
3250	IRLM1.G	-	+	+
	IRLM1	-	-	-
3300	IRLM1.G	-	-	-
	IRLM1	-	-	-

The second population of mutant strains (IRLM1.D) was achieved by treatment with DES (0.5% to 3% v/w). The number of colonies on the MSM agar containing Diazinon was decreased from 0.5% to 3% of DES treatment. The green pigment that is the characteristic of *Pseudomonas* was not generated at a rate of 2% and 2.5% of DES. The mutant strains were

selected based on the magnitude of halo on LB agar with monocrotophos and the rate of diazinon biodegradation. Therefore, eight mutant strains were considered as the proper choice for genome shuffling. Based on Table 2, the IRLM1.D4 and IRLM1.D8 strains demonstrate satisfying results than other strains. Thus, the IRLM1.D8 strain was considered as one of the parent strains suitable for genome shuffling. Figures 1 and 2 show the diazinon biodegradation at 2000 and 3000 mg/L, respectively. And also, the comparison of whole-cell activity is shown in Figure 3.

Table 2. The second population of mutant strains.

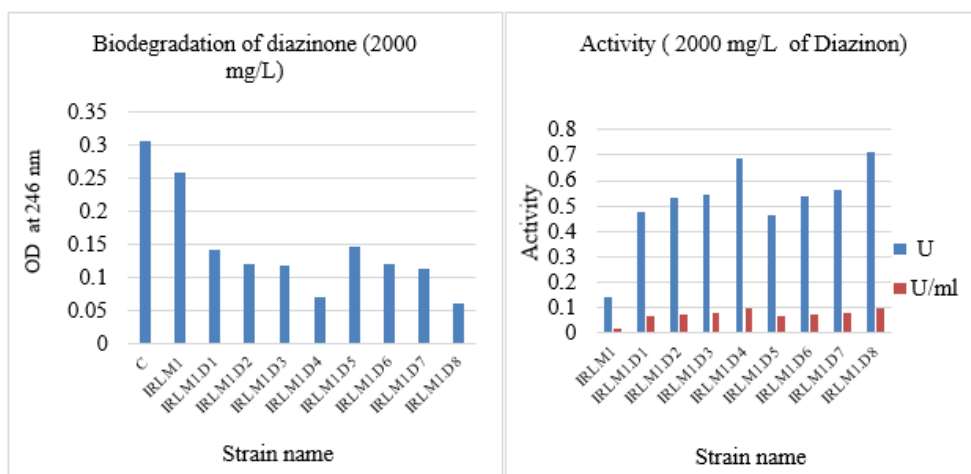| Strain name	OD at 246 nm (2000 mg/L)	OD at 246 nm (3000 mg/L)
C	0.306	1.679
IRLM1	0.257	1.656
IRLM1.D1	0.141	1.595
IRLM1.D2	0.121	1.655
IRLM1.D3	0.117	1.499
IRLM1.D4	0.069	1.432
IRLM1.D5	0.146	1.497
IRLM1.D6	0.12	1.486
IRLM1.D7	0.112	1.475
IRLM1.D8	0.061	1.313

Figure 1. Evaluation of diazinon biodegradation (2000 mg/L diazinon) by improved strains with DES mutagenesis process (A) and calculation of whole-cell activity (B). C: non-inoculated control; IRLM1: wild type strain; IRLM.Ds: starting strains.

Figure 2. Evaluation of diazinon biodegradation (3000 mg/L of Diazinon) by improved strains with DES mutagenesis process (A) and calculation of whole-cell activity (B). C: non-inoculated control; IRLM1: wild type strain; IRLM.Ds: starting strain.
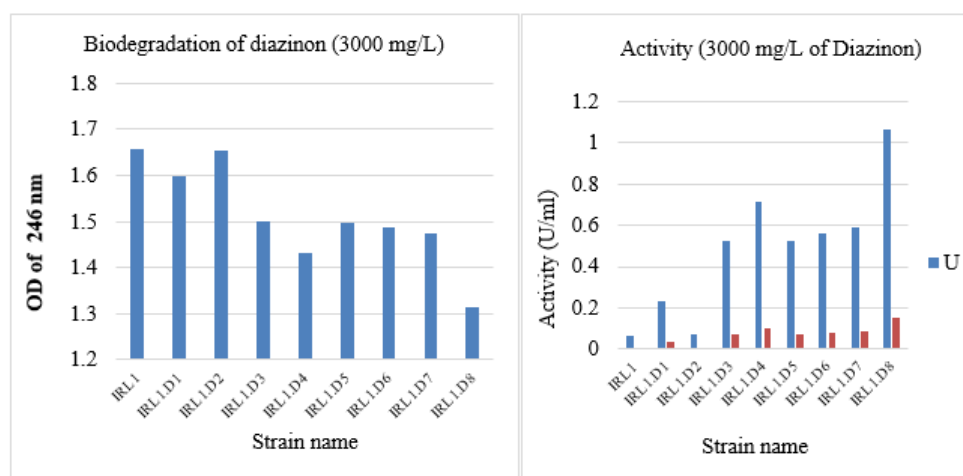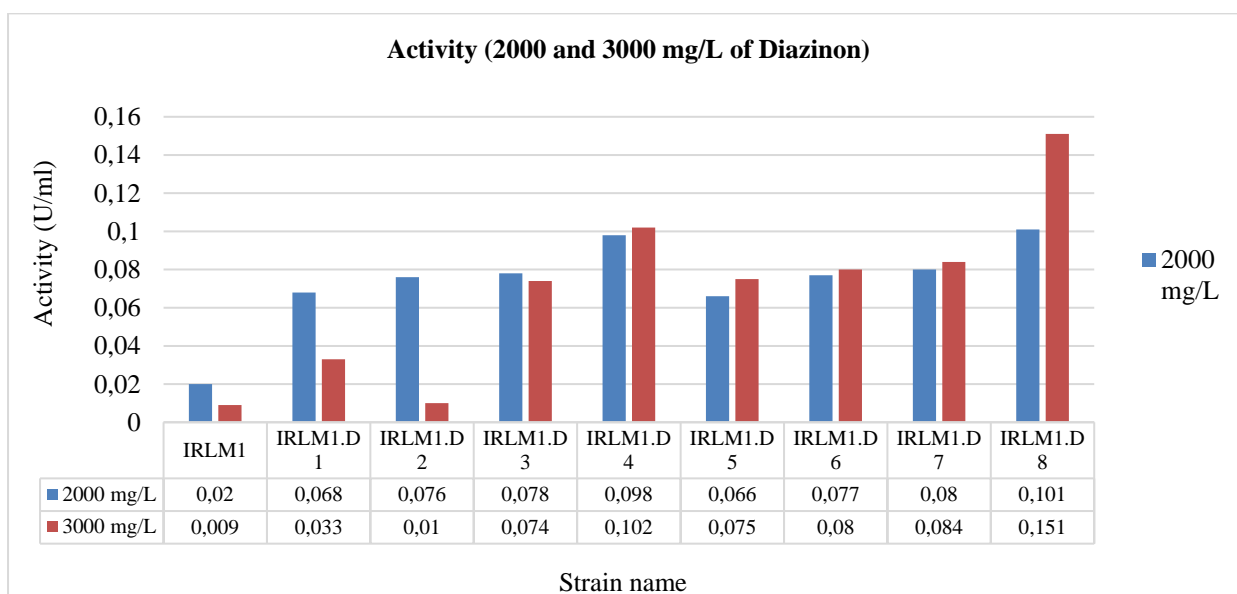
Activity (2000 and 3000 mg/L of Diazinon)

Strain name	2000 mg/L	3000 mg/L
IRLM1	0,02	0,009
IRLM1.D1	0,068	0,033
IRLM1.D2	0,076	0,01
IRLM1.D3	0,078	0,074
IRLM1.D4	0,098	0,102
IRLM1.D5	0,066	0,075
IRLM1.D6	0,077	0,08
IRLM1.D7	0,08	0,084
IRLM1.D8	0,101	0,151

Figure 3. Comparison of whole-cell activity by improved strains with DES in 2000 and 3000 mg/L of Diazinon.

3.2. Genome shuffling and screening.

The created protoplasts were observed by a light microscope after the breakdown of the cell wall with lysozyme (Figure 4). The shuffled strains were selected based on the magnitude of halo on LB agar with monocrotophos. The 12 shuffled strains were selected for analytical methods.
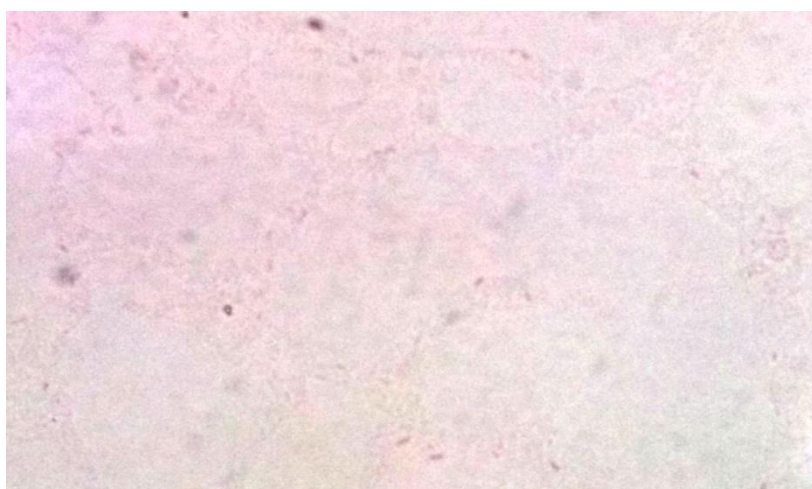

Figure 4. The prepared protoplasts.

3.3. Bacterial growth and biodegradation of Diazinon in MSM.

The biodegradation of Diazinon in MSM with Diazinon monitored by optical density (OD$_{246}$) and growth ability of shuffled strains was evaluated by its cultivation in MSM agar with Diazinon. The results showed significant differences (p<0.05) in the biodegradation rate of individual shuffled strains than parent strains (IRLM1.G and IRLM1.D) and control strains in MSM with Diazinon (Table 3). The results of growth on the MSM agar with Diazinon (3000, 4000 and 5000 mg/L) showed that only one of the shuffled strains (IRLM1.F3) was able to grow on 3000 mg/L diazinon, but only one of them was able to grow on 4000 and 5000 mg/L of Diazinon. In contrast, the native strain (IRLM1) showed few changes in OD$_{246}$ than the non-inoculated sample. Evaluation of diazinon biodegradation of shuffled strains is shown in Figure

5. And also, Figure 6 shows the activity of the whole-cell of shuffled strains. The growth of shuffled strains on MSM agar supplemented with Diazinon is presented in Table 4.

Table 3. Shuffled strains.

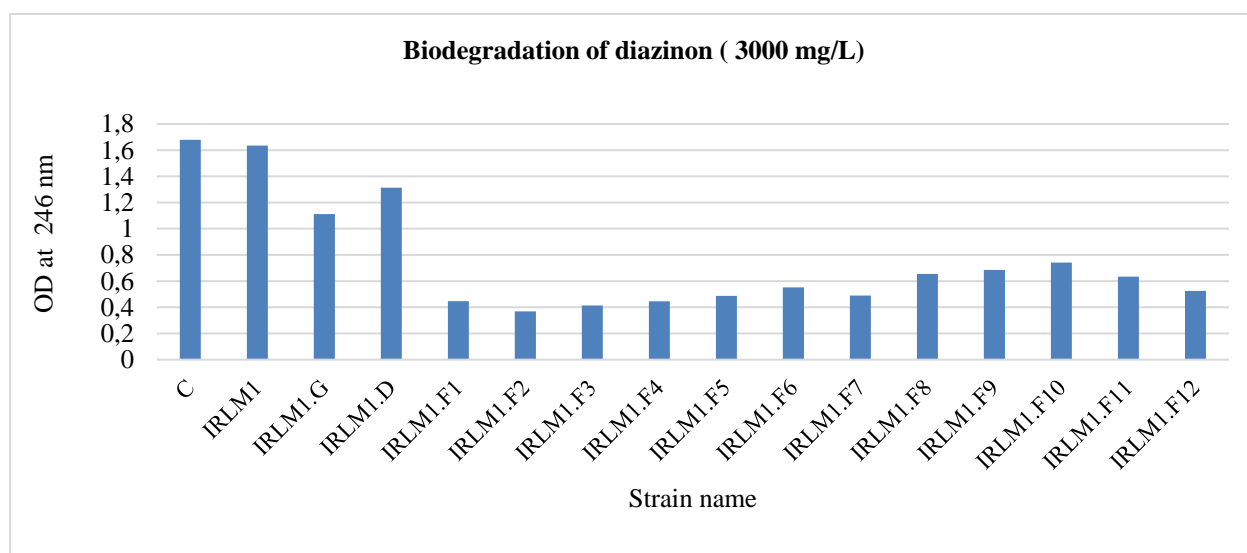| Strain name	OD $_{246 nm}$ (3000 mg/L)
C	1.679
IRLM1	1.635
IRLM1.G	1.112
IRLM1.D	1.313
IRLM1.F1	0.447
IRLM1.F2	0.369
IRLM1.F3	0.414
IRLM1.F4	0.446
IRLM1.F5	0.487
IRLM1.F6	0.552
IRLM1.F7	0.49
IRLM1.F8	0.654
IRLM1.F9	0.685
IRLM1.F10	0.741
IRLM1.F11	0.634
IRLM1.F12	0.525

Figure 5. Evaluation of diazinon biodegradation (3000 mg/L of Diazinon) by shuffled strains.
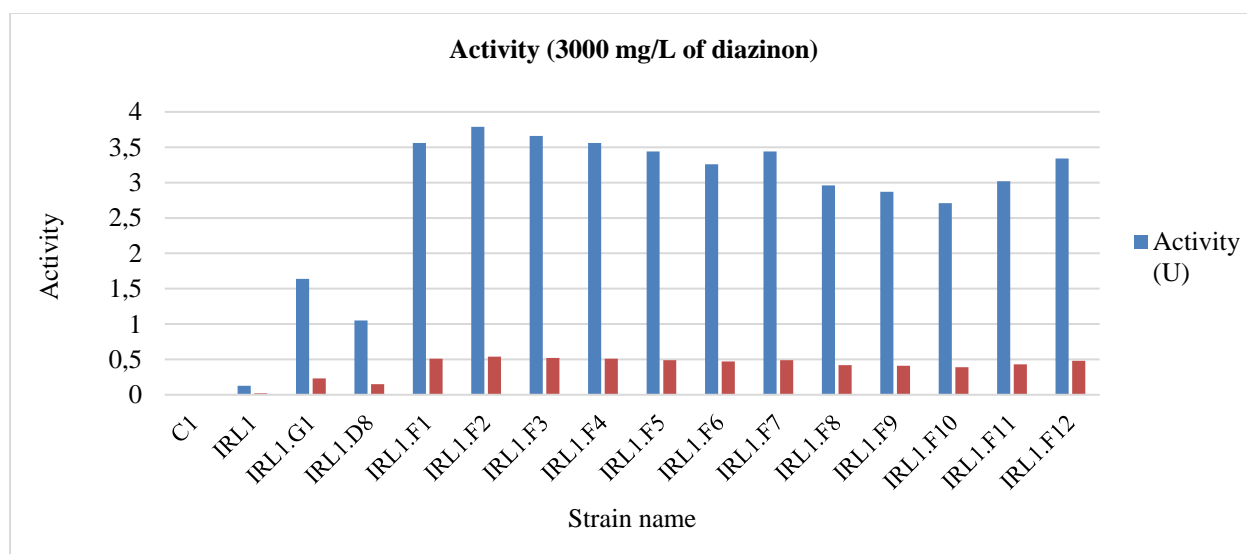

Figure 6. Evaluation of whole-cell activity by shuffled strains in 3000 mg/L of Diazinon.

Table 4. Growth of shuffled strains on MSM agar supplemented with Diazinon (in different concentrations).

Sample	Up to 2500 mg/L	3000 mg/L	4000 mg/L	5000 mg/L
IRLM1.G	+	+	-	-
IRLM1.D	+	-	-	-
IRLM1	+	-	-	-
IRLM1.F1	+	+	-	-
IRLM1.F2	+	+	+	+
IRLM1.F3	+	+	-	-
IRLM1.F4	+	+	-	-
IRLM1.F5	+	+	-	-
IRLM1.F6	+	+	-	-
IRLM1.F7	+	+	-	-
IRLM1.F8	+	+	-	-
IRLM1.F9	+	+	-	-
IRLM1.F10	+	+	-	-
IRLM1.F11	+	+	-	-
IRLM1.F12	+	+	-	-

Bioremediation of Op compounds from soil and surface water is an essential task. The *Flavobacterium sp*. ATCC 27551, the first bacterium to degrade Ops, was identified and isolated from the soil of the Philippine. So far, other bacteria have also been found that can use Op compounds as their sole source of carbon, nitrogen, or phosphorus. In various studies, strains of Op compounds have been introduced to clean up the environment. To remove Ops from industrial wastewater, Op degrading bacteria should be able to grow in high Op concentration, therefore, degrade these compounds. The wild type strain in the present study was isolated from soil and effluent storage pools of factories. While in the other study, the Op degrading bacteria had been isolated from the soil of fields. This is a strong point in this research due to our wild type strain, which can grow in higher concentrations of Diazinon (up to 2900 mg/L). Additionally, the activity of this wild type strain was improved by the genome shuffling method. Since this study was aiming to improve the bacteria activity and confer the ability to grow in higher Op concentration, Op adaptation and DES mutagenesis was applied. In comparison, in other studied Patnaik et al. (2002) [31] to improve the pH tolerance and acid production in bacteria, pH adaptation, and nitrosoguanidine (NTG) mutagenesis was used.

Some changes, such as the increase the tolerance to Ops and the ability to grow in the high concentration of Ops, require tremendous changes in the genome, which are not predictable. After Op adaptation, the IRLM1.G strain was able to tolerate and grow in 3250 mg/L of Diazinon. It can be concluded that a gradual increase in diazinon concentration may cause mutation, which leads to adaptation with the new condition. On the other hand, in many studies, DES mutagenesis has been used for the preparation of starting strains. Also, we observed that DES mutagenesis was efficient in obtaining the IRLM1.D8 strain as the improved starting strain. So that the whole-cell activity by the IRLM1D8 was up to 5 and 16.7 fold higher than the wild type strain in 2000 and 3000 mg/L of Diazinon, respectively. Many mutated strains with DES (IRLM1.F3, 4, 5, 6, and 7) showed an equal increase in biodegradation in 2000 and 3000 mg/L of Diazinon. Thus, the induced mutations in the genes involved in the decomposition of organophosphate compounds did not disrupt the degrading activity even at a high concentration of Diazinon.

After one round of genome shuffling and screening, all the strains showed remarkable whole-cell activity in 3000 mg/L diazinon so that the whole-cell activity by IRLM1.F2 and IRLM.F3 was up to 0.52 and 0.54 U/ml, respectively. This activity was 60 and 57.7 fold higher than the wild type strain and 3.5 and 3.4 fold higher than the starting strain. In the study by Wang Ch et al. (2013) [32] after 6 rounds of genome shuffling, 4.2 fold improvement for

Penicillium citrinum and in the study by Patnaik R et al. (2002) [31] after 5 rounds of genome shuffling 3 fold improvement for *Lactobacillus* was reported. Zhang Y. F. et al. (2014) [33] also obtained 2.4 fold improvement in *Lactococcus lactis* after 4 rounds. One of the profound differences between the present study and other studies is the number of genome shuffling rounds, which was performed in just one round compared to several rounds of genome shuffling in other studies. However, the choice of method plays an important role in the final result. In some methods, the outcome shows no difference between the wild type strain and the shuffled strain, and therefore, the enzyme activity remains the same or increases briefly.

So far, the other methods such as DNA shuffling and error-prone PCR [34] have been used to produce recombinant strains by several researchers, but the use of microorganisms in bioremediation has its advantages such as stability, adaptation to the environment, cost-effective and easy production. Several bacteria with almost identical *opd* sequences have been identified from around the world. The *opd* gene exists in the genome of several bacteria. The gene that is responsible for the breakdown of Diazinon and other Ops is *opd,* which also can be found in *Pseudomonas* Strain.

Although all fusants showed improved activity compared to the wild type, only IRLM1.F3 was able to grow in a media containing more than 3000 mg/L of diazinon concentration. Other strains, despite their increased activity, were not able to grow in higher concentrations of Diazinon. Nevertheless, the occurrence of both increased activity and the ability to grow in high concentrations of Diazinon is less likely. Thus, we successfully obtained the IRLM1.F3 strain with increased activity (57.7 fold higher than wild type strain) and the ability to grow in high concentrations (up to 5000 mg/L) of Diazinon after one round of genome shuffling.

4. Conclusions

The genome shuffling could easily and rapidly improve the characteristics of microorganisms to be used in commercial applications. Based on our results, the IRLM1.F2 strain was considered as the candidate strain for industrial production in bioremediation purposes, especially in wastewater treatment. It can also be used for the characterization of the mutations responsible for improved activity and tolerance.

Funding

Acknowledgments

Conflicts of Interest

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