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Genetic Modification of Alkaline Protease, Lipase Activities, SDS-PAGE Proteins and Other Characters in Some Bacterial Strains

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Abstract: Gram-positive *Staphylococcus aureus*, *Bacillus subtilis* and Gram-negative *Pseudomonas fluorescens* were cured from their own plasmids using an elevated temperature or acridine orange. Elevated temperature was more efficient than acridine orange. Plasmid profiles were varied in size, number and their existence. However, B3 cured strain resulted of *B. subtilis* by elevated temperature changed their sensitivity profile and became resistant to kanamycin and tetracycline. Similar change was found in P1, P2 and P3 cured strains resulted of *P. fluorescens* by the two curing agents and became resistant to ampicillin and chloramephincol. The relationship between the presence of plasmid and their antimicrobial resistance was detected in cured strains by acridine orange. Whereas, S1 and S2 cured strains resulted of *S. aureus* that harboring three plasmids of its original strain were altered their resistance and become sensitive to kanamycin and rifampicin. B1 and B2 cured strains that harboring two plasmids of its original strain showed the similar manner to neomycin. On the other hand, elevated temperature severely affected the enzyme productivity in cured strains and thereby loss their activities while hyperactivities were observed by acridine orange. For instance, S1 and S2 cured strains recorded 14.9 and 10.7 U mL^{-1} of alkaline protease while the original strain lacked the ability to produce the enzyme. In contrast, B1 cured strain revealed hyperactivity that reached around 14-fold increase in lipase activity with 102.3 U mL^{-1} than the original strain whereas S1 cured strain reached around 4.2-fold increase with 192 U mL^{-1} and P1 cured strain reached around 2-fold increase with 69.3 U mL^{-1}. S3 and B3 cured strains were reduced one and two protein bands comparing with the other produced by acridine orange. However, P3 cured strain revealed high total protein bands similar to the original strain. Moreover, the newly induced protein bands were higher in the cured strains by temperature than acridine orange.

Key words: Alkaline protease and lipase activities, antibiotic susceptibility, bacterial strains, plasmid curing, SDS-PAGE proteins

INTRODUCTION

Lipases (triacylglycerol acylhydrolases) are an important group of biotechnologically relevant enzymes that find immense applications in various areas of industrial microbiology and biotechnology such as food and dairy, detergent and pharmaceutical (Sharma *et al*., 2001). Microbial lipases play a vital role in commercial ventures, whereas some important lipase-producing bacterial genera include *Bacillus*, *Pseudomonas* and *Staphylococcus* sp.

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(Bora and Kalita, 2007). Lipases are generally produced on lipidic carbon, such as oils, fatty acids, glycerol or tweens in the presence of an organic nitrogen source (Lee *et al*., 2006). Moreover, proteases constitute one of the most important groups of industrial enzymes that account for about 60% of the total worldwide enzymes sales for their wide temperature and pH tolerance and stability (Genckal and Tarib, 2006). They can hydrolyze proteins into short peptides or amino acids. *Bacillus* species are prolific producers of extra cellular proteases that have a wide range of applications, particular in the detergent, food, pharmaceutical, leather and chemical industries (Zhang *et al*., 2008).

Bacteria possess ensembles of transcriptionally regulated genes, commonly called stress or shock response systems that enable them to adapt very rapidly to changes in the chemical or physical aspects of their environment, including water activity, pH, temperature and oxygen concentration (Wood *et al*., 2001). Gunasekera *et al*. (2008) reported that adaptation to environmental shifts involves a transient or acute phase (shock response) that consists of rapid responses needed to initiate the adaptation to the new conditions and a continuous or chronic phase (stress response), possibly at a new growth rate, in the altered environment. Woods *et al*. (2001) found that extracellular lipase and protease production by *Pseudomonas fluorescens* B52 is repressed by iron and regulated by temperature, whereas lipase production is increased below the optimum growth temperature (low-temperature regulation), while protease production was relatively constant and only decreased above the optimum growth temperature (thermoregulation).

Several microorganisms including *Pseudomonas* sp. and *Bacillus* sp. have been studied for their lipid degradation ability on a laboratory scale in efforts to improve the biodegradation of oils and fats in wastewater (Matsuoka *et al*., 2009). Limitations of the industrial use of the lipase and protease have mainly been due to their high production costs, which may be overcome by molecular technologies, enabling the production of these enzymes at high levels and in a virtually purified form (Houde *et al*., 2004).

Plasmids DNA have a major impact on metabolic function. Varieties of physical and chemical curing agents were used to cure bacteria and obtain strains lacking plasmids. Plasmids are eliminated by these agents because of interference with their replication; DNA intercalating dyes (acridine orange) and by alterations of their membrane attachment sites (elevated temperature). Plasmid elimination *in vitro* provides a method of isolating plasmid free bacteria for biotechnology without any risk of inducing mutations (Spengler *et al*., 2006). Jaiswal and Singh (1990) proved that the virulence in *Clostridium perfringens* type B was affected by plasmid curing as a result of the decrease in toxin production while Beg and Ahmad (2000) proved that curing of plasmid pUK 651 from *E. coli* x^+ was confirmed by determining the loss of resistance markers in the cured derivative culture.

The aim of the present study was to produce new modified bacterial strains with more efficient and enhanced alkaline protease and lipase enzymes productivity and activity as well as other characters, such as antibiotic susceptibility and SDS-PAGE total proteins using plasmid curing with elevated temperature and acridine orange treatment. Such new modified strains can be used for economic industries and for successive genetic improvement programs.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

A local *Bacillus subtilis* strain and two bacterial strains; *Staphylococcus aureus* and *Pseudomonas fluorescens* obtained from (American Type Culture Collection, USA) were used in the study. LB broth and LB agar media (Davis *et al*., 1980) were used to grow the strains at 37°C with vigorous aeration.

Plasmid Isolation and Curing

Plasmids of the three bacterial strains were isolated using mini preps method of Rodriguez and Tait (1983). Electrophoresis was performed using 0.7% agarose gel. The three bacterial strains were cured from their own plasmids using two treatments, they growing overnight at 37°C in broth culture supplemented with 100 μg mL^{-1} acridine orange dissolved in absolute ethanol (Ramteke and Tewari, 2007) and growing at elevated temperatures 43°C for two days. Appropriate dilutions of each curing treatment were spread on LB plates and incubated at 37°C. Four random single colonies of each strain were selected in each treatment and tested for their sensitivity against tetracycline (Tc) (Garrity, 2001) and for any variations in their alkaline protease and lipase productivities.

Antimicrobial Susceptibility

Seven antibiotics were used with final concentrations in (μg mL^{-1}) according to Dionisio *et al.* (2002) as follows: ampicillin (Amp) 100, chloramephincol (Cm) 35, streptomycin (Sm) 200 procured from Bio Basic Inc. (Canada), tetracycline (Tc) 15, rifampicin (Rif) 100, kanamycin (Km) 40 (Sigma, St. Louis, USA) and neomycin (Nm) 40 (Fluka BioChemika Switzerland). Tetracycline was dissolved in acetic acid and ethanol and diluted in demonized H_2O to the required concentration. Rifampicine were dissolved in phosphate-buffered saline (PBS) pH 7.2 and the others were dissolved in demonized H_2O. The antibiotics were sterilized through 0.45 μm pore-size filters (Millipore S.A., France). The Kirby-Bauer disc diffusion method for antimicrobial susceptibility test was used (National Committee for Clinical Laboratory Standards, 2000).

Alkaline Protease Activity and Production Assay

Alkaline protease production was determined on LB agar plate according to Adinarayana *et al.* (2003). The LB agar contained 1% skim milk, 1% tryptone (Difco), 0.5% yeast extract (Gibco BRL), 0.5% NaCl and 1.5% agar. The three bacterial strains were grown on the agar plates at 37°C for 40 h to detect the enzyme production, where clear zone of skim milk hydrolysis gave an indication of enzyme production under alkaline conditions. Enzyme activity was determined according to Dumusois and Preist (1993) using azocasein as a substrate. One milliliter of 0.4% azocasein in 0.1 M Tris-HCl buffer pH 8.5 was added to 0.5 mL enzyme source, the reaction mixture was incubated at 40°C for 30 min and then 1 mL of 15% trichloroacetic acid (TCA) was added. After centrifugation, absorbance of clear supernatant was measured at 420 nm. One enzyme unit was defined as the amount of enzymes that gives an increase of 0.1 OD at 420 nm under certain reaction conditions.

Lipase Activity and Production Assay

Lipase production was determined using LB agar plates plus 0.5% tributyrin (Sigma) according to Krzeslak *et al.* (2008), whereas enzyme production forms halos around colonies. Enzyme activity was determined using Tween-20 as a substrate according to Von Tigerstrom and Stelmaschuk (1989) using a mixture of 2% Tween-20 in 20 mM Tris-HCl buffer pH 7, 120 mM $CaCl_2$ and 0.3 mL of enzyme source. The reaction was followed periodically by measuring the increase in optical density at 500 nm due to the hydrolytic release of the fatty acids from Tween-20 and their precipitation as the calcium salts. The reaction was carried out for a period of 30 min at 37°C. One enzyme unit was defined as the amount of enzyme that release fatty acid-calcium complex equivalent to 0.01 OD at 500 nm under standard reaction conditions.

SDS-PAGE Protein Analysis

The three bacterial strains with their plasmid-cured colonies were grown in suspension following the method of Alberola *et al.* (1999). One hundred milliliter of nutrient broth was inoculated into 500 mL flasks with one loop of bacteria and shaken for 3 days at 30°C (220 rpm). The suspension was centrifuged for 10 min at 10,000 rpm at 4°C; the pellet was washed twice in water and resuspended in 4 mL of water. The supernatants were autoclaved (121°C for 10 min) and the colonies were resuspended into 1 mL of ice-cold 0.5 M NaCl. The cells were centrifuged at 13,000 rpm for 5 min and the pellet was resuspended in 1% SDS, 0.01% β-mercaptoethanol, boiled for 10 min and recentrifuged at 13,000 rpm for 10 min. The supernatant was removed and analyzed by 15% SDS-PAGE according to Von Tersch and Gonzalez (1994).

RESULTS

Plasmid Profiles

The plasmid profiles of the original and cured strains presented different patterns as shown in Fig. 1. Moreover, the plasmids of the original strains were varied in size, number and their existence as shown in Table 1. For example, *S. aureus* displayed the highest number of plasmids with seven, followed by *B. subtilis* with five, while *P. fluorescens* showed the lowest number with three. Among the cured strains of each original strain via elevated temperature showed the lowest numbers. For instance, S3 displayed the highest number with two, followed by B3 with one and P3 was plasmids free. Moreover, the two cured strains resulted of *S. aureus* by acridine orange showed three different plasmids, while in the other two strains showed two plasmids with different molecular sizes. For example, P2 cured strain via acridine orange resulted of *P. fluorescens* displayed the similar two plasmids in the original strain with sizes 1095 and 666 bp, while one plasmid with 1662 bp was eliminated. The cured strain P3 via elevated temperature lost the three plasmids presented in its original strain. The three cured strains resulted of *S. aureus* showed two similar plasmids like the

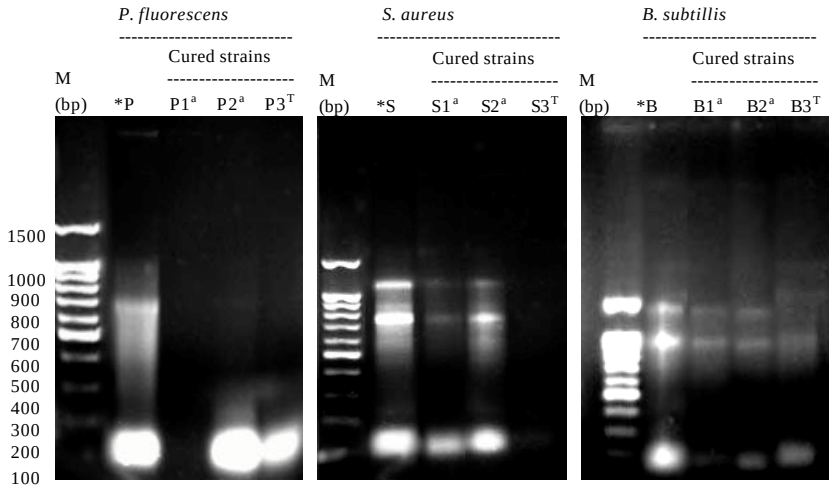

Fig. 1: Plasmid profile of the original strains and their cured strains via acridine orange ([a]) or elevated temperature ([T]). M = DNA ladder with 100 bp and * = Original bacterial strains

Table 1: Plasmid numbers corresponding to their sizes in the three bacterial strains and their plasmid-cured strains

		P. fluorescens				*S. aureus*				*B. subtilis*			
			Cured				Cured				Cured		
Plasmid No.	Plasmid size (bp)	*P	P1[a]	P2[a]	P3[T]	*S	S1[a]	S2[a]	S3[T]	*B	B1[a]	B2[a]	B3[T]
1	3135									+	+	+	+
2	1740					+	+	+	+				
3	1662	+											
4	1491									+	+	+	
5	1154					+	+	+	+				
6	1095	+		+									
7	888					+		+					
8	833									+			
9	734					+	+						
10	666	+		+									
11	561									+			
12	555					+							
13	439					+							
14	271									+			
15	209					+							
Total numbers		3	0	2	0	7	3	3	2	5	2	2	1

*: Original bacterial strains, a: Cured by acridine orange, T: Cured by elevated temperature

Table 2: Antibiotics resistant patterns of the three bacterial strains and their plasmid-cured strains using seven antibiotics

		Antibiotics							
Original strains	Cured strains	Km	Tc	Rif	Sm	Amp	Cm	Nm	Plasmid No.
P. fluorescens (*P)		-	-	-	-	-	-	-	3
	P. fluorescens (P1)[a]	-	-	-	-	+	+	+	0
	P. fluorescens (P2)[a]	-	-	-	-	+	+	-	2
	P. fluorescens (P3)[T]	-	-	-	-	+	+	-	0
S. aureus (*S)		+	-	+	-	+	+	+	7
	S. aureus (S1)[a]	-	-	-	-	+	+	+	3
	S. aureus (S2)[a]	-	-	-	-	+	+	+	3
	S. aureus (S3)[T]	-	-	+	-	+	+	+	2
B. subtilis (*B)		-	-	-	-	+	+	+	5
	B. subtilis (B1)[a]	-	-	-	-	+	+	-	2
	B. subtilis (B2)[a]	-	-	-	-	+	+	-	2
	B. subtilis (B3)[T]	+	+	-	-	+	+	+	1

+: Resistant (^{r}), -: Sensitive (^{s}), *: Original bacterial strains, [a]: Cured by acridine orange, [T]: Cured by elevated temperature

original strain with 1740 and 1154 bp. However, one plasmid with 888 bp that presented in the original strain was displayed in S2 and another one with 734 bp was displayed in S1. Three plasmids with low molecular sizes (555, 439 and 209 bp) were eliminated in the three cured strains resulted of *S. aureus*. The three cured strains resulted of *B. subtilis* showed one similar plasmid with 3135 bp like their original strain. One plasmid with 1491 bp was observed in cured strains B1 and B2 by acridine orange like their original strain *B. subtilis*, while B3 resulted by elevated temperature lost it. In addition, cured strains B1, B2 and B3 lost the three plasmids with low molecular sizes 833, 561 and 271 bp.

Alternation of Antibiotic Susceptible Patterns

The data in Table 2 shows the antibiotic resistant patterns of the three bacterial strains and their plasmid-cured strains produced by acridine orange and high temperature. The original *B. subtilis* strain was resistant to ampicillin (Amp^r), chloramephincol (Cm^r) and neomycin (Nm^r), while it was sensitive to the other four antibiotics. However, the cured strain (B3) via high temperature acquired the resistant to kanamycin (Km^r) and tetracycline (Tc^r) and the other two cured strains (B1 and B2) by acridine orange were changed their resistance profile and become sensitive to neomycin (Nm^s). *S. aureus* strain characterized by their

Table 3: Alkaline protease and lipase activities of the three bacterial strains and their plasmid-cured strains

Original strains	Cured strains	Alkaline protease activity	Lipase activity
		(Unit mL^{-1})	
P. fluorescens (*P)		13.3	35.3
	P. fluorescens (P1)[a]	10.6	69.3
	P. fluorescens (P2)[a]	2.4	0.0
	P. fluorescens (P3)[T]	0.0	0.0
S. aureus (*S)		0.0	46.2
	S. aureus (S1)[a]	14.9	192
	S. aureus (S2)[a]	10.7	0.0
	S. aureus (S3)[T]	0.0	0.0
B. subtilis (*B)		11.2	7.3
	B. subtilis (B1)[a]	4.0	102.3
	B. subtilis (B2)[a]	4.2	0.0
	B. subtilis (B3)[T]	0.0	0.0

*: Original bacterial strains, [a]: Cured by acridine orange, [T]: Cured by elevated temperature

resistance to five antibiotics and it was sensitive to tetracycline (Tc^s) and streptomycin (Sm^s), while the three cured strains (S1, S2 and S3) changed their resistance and become sensitive to kanamycin (Km^s) and two of them (S1 and S2) resulted by acridine orange become sensitive to rifampicin (Rif^s). *P. fluorescens* showed high sensitivity to all the seven antibiotics. It is interesting to note that the three cured strains changed their sensitivity profiles and became resistant to ampicillin (Amp^r) and chloramephincol (Cm^r) while P1 cured strain resulted by acridine orange turned into resistant to neomycin (Nm^r).

Hyperactivity of Lipase and Alkaline Protease in Cured Strains via Acridine Orange

Alkaline protease activities (U mL^{-1}) were varied considerably among the three original strains and their plasmid cured strains; however the cured strains P3, S3 and B3 resulted of *P. fluorescens*, *S. aureus* and *B. subtilis*, respectively, by elevated temperature were unable to produce the enzyme and recorded zero as shown in Table 3.

Hyperactivity of alkaline protease was obtained in the two cured strains S1 and S2 resulted of *S. aureus* by acridine orange that recorded 14.9 and 10.7 (U mL^{-1}), respectively while the original strain lacked the ability to produce the enzyme (Table 3). In contrast, *B. subtilis* was higher two and half times than their two cured strains B1 and B2. In the same manner *P. fluorescens* was higher than their cured strains.

On the other hand, the original strains displayed remarkable differences in lipase activity, whereas *S. aureus* showed the highest activity with 46.2 (U mL^{-1}), followed by *P. fluorescens* with 35.3, while *B. subtilis* showed the lowest with 7.30 as shown in Table 3. However, the cured strains P3, S3 and B3 resulted of *P. fluorescens*, *S. aureus* and *B. subtilis*, respectively by elevated temperature were unable to produce the enzyme and recorded zero (U mL^{-1}) as shown in Table 3. In contrast, one of each cured strains obtained via acridine orange revealed hyperactivity. B1 cured strain resulted of *B. subtilis* revealed hyperactivity that reached around 14-fold increase in activity than its original strain with 102.3 (U mL^{-1}) whereas S1 cured strain resulted of *S. aureus* reached around 4.2-fold increase with 192 U mL^{-1} and P1 cured strain resulted of *P. fluorescens* reached around 2-fold increase with 69.3 U mL^{-1} as shown in Table 3. However, the remaining cured strains were unable to produce any lipase activity and they estimated zero (U mL^{-1}).

Protein Polymorphism

The protein bands of the original and cured strains were categorized in three group types according to their appearance profiles among samples as shown in Fig. 2 and Table 4. Type 1 displayed bands that disappeared in the original strains and appeared in their cured strains, whereas the high numbers with 6 and 7 bands appeared in cured strains, P2 and P3

Table 4: Protein analysis of the three bacterial strains and their plasmid-cured strains

	P. fluorescens						*S. aureus*						*B. subtilis*					
				Cured strains						Cured strains						Cured strains		
Group type	Band No.	MW (kDa)	*P	P1[a]	P2[a]	P3[T]	Band No.	MW (kDa)	*S	S1[a]	S2[a]	S3[T]	Band No.	MW (kDa)	*B	B1[a]	B2[a]	B3[T]
1	3	174				+		5	121			+	26	17				+
	1	186			+	+		11	84			+	30	11			+	
	16	74			+	+		13	67	+	+	28	14				+	+
	20	55			+	+		22	27	+	+		1	177		+	+	+
	22	48			+	+							10	103		+	+	+
	26	34			+	+							24	23		+	+	+
	6	146		+	+	+							32	7		+	+	+
	Total	0	1	6	7			0	1	2	3				0	4	6	6
2	35	10	+					30	10	+								
	37	8	+					33	7	+								
	8	122	+															
	Total	3	0	0	0			2	0	0	0				0	0	0	0
3	14	88	+	+				15	62	+		+	4	135	+	+		
	31	17	+	+				24	21	+		+	12	92	+	+		
	36	9	+	+				1	180	+	+	+	17	59	+	+		
	5	155	+	+	+			6	114	+	+	+	2	155	+	+	+	
	4	162	+	+		+		23	24	+	+	+	7	119	+	+	+	
	9	119	+		+	+		28	12	+	+	+	11	98	+	+	+	
	13	93	+		+	+		29	11	+	+	+	15	75	+	+	+	
	15	79	+		+	+							21	39	+	+	+	
	19	59	+		+	+							27	15	+	+	+	
	23	45	+	+		+							6	124	+		+	
	33	12	+	+		+							3	145	+		+	+
													8	112	+	+		+
													9	108	+	+		+
													18	54	+	+		+
													19	50	+	+		+
													25	20	+		+	+
													29	12	+		+	+
													31	9	+		+	+
	Total	11	7	5	7			7	5	5	2		18	13	10	9		
4	2	178	+	+	+	+	2	170	+	+	+	+	5	132	+	+	+	+
	7	136	+	+	+	+	3	146	+	+	+	+	13	84	+	+	+	+
	10	114	+	+	+	+	4	133	+	+	+	+	14	79	+	+	+	+
	11	109	+	+	+	+	7	111	+	+	+	+	16	64	+	+	+	+
	12	100	+	+	+	+	8	106	+	+	+	+	20	46	+	+	+	+
	17	71	+	+	+	+	9	96	+	+	+	+	22	30	+	+	+	+
	18	66	+	+	+	+	10	92	+	+	+	+	23	26	+	+	+	+
	21	52	+	+	+	+	12	75	+	+	+	+	33	6	+	+	+	+
	24	41	+	+	+	+	14	66	+	+	+	+						
	25	37	+	+	+	+	16	57	+	+	+	+						
	27	30	+	+	+	+		17	52	+	+	+	+					
	28	26	+	+	+	+		18	46	+	+	+	+					
	29	23	+	+	+	+		19	43	+	+	+	+					
	30	21	+	+	+	+		20	33	+	+	+	+					
	32	15	+	+	+	+		21	30	+	+	+	+					
	34	10	+	+	+	+		25	19	+	+	+	+					
	38	7	+	+	+	+		26	16	+	+	+	+					
								27	13	+	+	+	+					
								31	9	+	+	+	+					
								32	8	+	+	+	+					
Total	17	17	17	17						20	20	20	20		8	8	8	8
Total number =		38	31	25	28	31		33	29	26	27	25		33	26	25	24	23

*: Original bacterial strains, [a]: Cured by acridine orange, [T]: Cured by elevated temperature

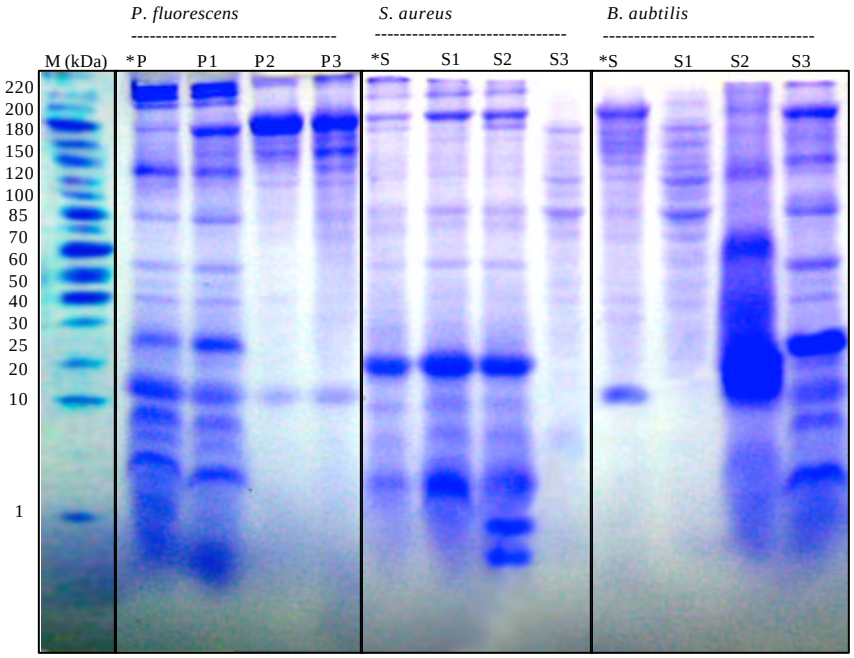

Fig. 2: SDS-PAGE protein analysis of the original strains and their plasmids-cured strains. * = Original bacterial strains and the other referred to the cured strains. M = Protein molecular weight (kDa)

resulted of *P. fluorescens*, 2 and 3 bands in cured strains, S2 and S3 resulted of *S. aureus* and 6 bands in cured strains, B2 and B3 resulted of *B. subtilis*. In contrast, type 2 displayed the bands that existed in the original strains and disappeared in the cured strains, where 3 bands with (8, 10 and 122 kDa) in *P. fluorescens* and 2 bands with (7 and 10 kDa) in *S. aureus*. The type 3 showed the bands that existed in the original strains as well as in some cured strains and the most commonly detected bands in all samples were grouped in type 4. The total protein band numbers were varied in the original strains comparing with the cured strains and even between the cured strains in each strain. For example, a total of 38 bands were detected in *P. fluorescens* and 33 bands in both of *S. aureus* and *B. subtilis*. However, the original strains mostly displayed higher total numbers than the cured strains as shown in Table 4.

DISCUSSION

It is clearly observed from Fig. 1 and Table 1 that elevated temperature cured more plasmids than acridine orange among the tested strains. However, plasmid curing by elevated temperature in *B. subtilis* changed the sensitivity profile, became resistant to kanamycin (Km^r) and tetracycline (Tc^r) and displayed unique resistant pattern comparing with the cured strains obtained by acridine orange in such strain. While, cured strains by elevated temperature displayed the similar antibiotic patterns in the two other strains comparing with acridine orange. Similar result was reported by Kulkarni and Kanekar (1998); where elevated temperature alone at 40°C was ineffective in curing the only single plasmid exist in strain of *Paseudomonas putida* that capable of utilizing caprolactam as a sole source of carbon and nitrogen, while acridine orange, ethidium bromide and SDS failed to cure the plasmid.

On the other hand, the relationship between the presence of plasmid and their antimicrobial resistance was detected in the cured strains by acridine orange. Whereas, S1 and S2 that harboring three of plasmids presented in *S. aureus* were altered their resistance and become sensitive to kanamycin (Km^s) and rifampicin (Rif^s) and B1 and B2 that harboring two of plasmids presented in *B. subtilis* showed the similar manner to neomycin (Nm^s) as shown in Table 2.

The results were agreed with Luque *et al.* (1994), who found that curing of extrachromosomic elements by acridine orange showed a percentage of resistance lost greater than 70% for kanamycin and neomycin. They indicated that the resistance to those antibiotics is mainly linked to plasmids and suggested more studies to demonstrate the direct association between antimicrobial resistance and presence of plasmids. Twelve isolates of *Klebsiella pneumoniae* were resistant to gentamicin, kanamycin, tetracycline and chloramphenicol but sensitive to cefoxitin, imipenem, amikacin and tobramycin.They found that all isolates carried an identical plasmid of 87 kb and resistance to â-lactams, aminoglycosides, tetracycline and chloramphenicol was lost after plasmid curing by acridine orange (Araque *et al*., 2000).

However, Nagy *et al.* (1990) reported no correlation between plasmid harboring and resistance against ten antibiotics in 26 *Bacteroides fragilis* group strains and no curing of the strains from the plasmids was achieved with acridine orange. Attempts to eliminate the plasmid from *Chlamydia trachomatis* strain L1 440 using the plasmid-curing agent acridine orange led to a paradoxical increase in plasmid copy number. It is speculated that the stress induced by chemical curing agents may stimulate the activity of plasmid-encoded replication (Rep) proteins (Pickett *et al*., 2005).

The effects of acridine orange in plasmid curing have been reported in different species. For instance, Macinga and Rather (1999) reported that the regulation of *aac(2')-Ia* in *Providencia* strains expression is extremely complex involving at least seven regulatory genes acting in at least two pathways. This complexity in regulation indicates that *aac(2')-Ia* expression must be tightly controlled in response to different environmental conditions. Moreover, they showed that the inability to eliminate the resistance phenotype from *Providencia* strains by curing with acridine orange, led to suggestion that the *aac(2')-Ia* gene was chromosomally encoded. Another study by Raja and Selvam (2009) revealed that wild type isolate *Pseudomonas aeruginosa* BC15 and transformant were resistant to ampicillin, while cured and *E. coli* DH5α were sensitive to ampicillin. From this data, the genes encoding resistance to ampicillin are located on the plasmid pBC15. Furthermore, wild type BC15 was resistant to tetracycline, chloramphenicol, streptomycin, kanamycin and erythromycin. However, cured strain was sensitive to these antibiotics indicating the possible plasmid borne nature of genes encoding resistance to these antibiotics. This suggested that loss of antibiotic resistance phenotype in cured strain may be either because of mutation because of incubation in the presence of the curing or genes encoding resistance to the above antibiotics are not transferred to *E. coli*. Conversely, Comai and Kosuge (1980) proved that loss of capacity to produce indole-3-acetic acid (IAA) of *Pseudomonas savastanoi* cause olive knot disease symptoms was obtained by acridine orange treatment and this was correlated with loss of a plasmid called pIAA1. Misra *et al.* (1988) showed no significant reduction in specific activity of three enzymes; CO dehydrogenase, formate dehydrogenase and hydrogenase in cured strains due to the loss of the plasmids in *Clostridium thermoaceticum* cells treated with acridine orange.

On the other hand, Woods *et al.* (2001) compared the profile of lipase production with that of protease with respect to temperature. The production of maximal levels of lipase at

temperatures below the optimum growth rate showed the classical pattern (Andersson, 1980); with a level of activity, six fold greater at 17°C than at 27°C, confirming the low-temperature regulation for strain *P. fluorescens* B52. The production of protease in the same culture showed a different pattern; production was very similar at 17 and 27°C and only decreased when the growth rate declined above 27°C (McKellar and Cholette, 1987). This differs from the pattern observed by Gugi *et al.* (1991) for *P. fluorescens* MF0 although, the difference in protease levels between 17 and 30°C was only about twofold. Woods *et al.* (2001) concluded that low temperature regulation in *P. fluorescens* B52 is exemplified by lipase production and that the decreased production of protease above the optimal growth temperature may be related to the decreased growth rate. Rashid *et al.* (2001) found that *Pseudomonas* sp. strain KB700A produced an extracellular lipase when grown in liquid medium at temperatures below 25°C, while no activity could be detected when cells were grown at 30°C either with or without Tributyrin. On the other hand, clear zone formation was observed around the KB700A colonies when grown at 30°C on Tributyrin agar plates, indicating that the gene could be expressed at this temperature. The absence of lipase activity in the culture supernatant at 30°C is likely to be due to the thermolability of the enzyme. In support of this, they have observed that KB-Lip was much more thermolabile than previously reported lipases.

Burger *et al.* (2000) showed that the production of protease by *P. fluorescens* LS107d2 at 29°C is dependent on PrtR, which they proposed is a novel member of a group of anti-sigma factors and transmembrane activators which interact with ECF (extracytoplasmic function) sigma factors of the σ^{70} family. In Pseudomonas fluorescens, the thermostable lipase (TliA) and protease genes are located upstream and downstream of ABC transporter operon (Ahn *et al.*, 2001). The tliDEF was different in gene organization and amino acid sequence homology (~50%) with aprDEF of P. aeruginosa. Homologous expression of tliDEF and tliA in P. fluorescens by plasmid-mediated supplementation of these genes enhances the lipase content over 1000 times than the original P. fluorescens and 100 times more than E. coli harboring tliDEFA (Chung *et al.*, 2009). A plasmid-free strain, *L. lactis* subsp. *cremoris* BC101, produces cell envelope-associated protease that is very similar or identical to the envelope protease encoded by the plasmid-linked *prtP* gene in other strains such as Wg2 and SK11. The *prtP* and *prtM* genes in this plasmid-free strain were identified on chromosomal DNA by pulsed-field gel electrophoresis (Nissen-Meyer *et al.*, 1992). The chromosomal protease gene was shown to be organized in a fashion similar to that of the plasmid-linked protease gene.

According to the molecular weights of lipase ad alkaline protease bands in many different reports, it is interesting to note that the identified molecular weights of lipase ad alkaline protease bands in the three strains shown in Fig. 2 and Table 4 were corresponding with similar weights to the same enzyme bands in such reports. For instance, protein analysis of lipase bands (Fig. 2, Table 4) showed three with (30, 52 and 66 kDa) in *P. fluorescens*, three with (30, 43 and 75 kDa) in *S. aureus* and two bands with (30 and 75 kDa) in *B. subtilis*. Moreover, alkaline protease bands revealed two with (15 and 20 kDa) in *B. subtilis*. Such obtained results were confirmed by Kawasaki *et al.* (2002), who reported that bacterial lipases are generally range in size from about 30 to 75 kDa. However, they reported that *B. subtilis* extracellular lipase has an exceptionally low molecular weight with 19.4 kDa for a member of the lipase family. It has been found that lipase from *P. fluorescens* (PFL) is able to aggregate into bimolecular structures 66 kDa even at moderate enzyme concentrations. At very low enzyme concentrations and in the presence of detergents, the same enzyme displayed a

unimolecular structure with a molecular weight of 33 kDa. Both enzyme structures displayed different functional properties (Fernández-Lorente *et al*., 2003). In order to compare lipases from two different *S. aureus* strains (FN 37 and TEN 5), the enzymes from the respective strains were purified and characterized. Differences in the size of the lipases in their native forms necessitated modifications of the purification process, but after purification identical subunits of about 43 kDa were found in SDS-PAGE and both lipases had an apparent molecular weight of 110 kDa when subjected to gel chromatography on Sephadex G-200 (Rollof *et al*., 1989). On the other hand, an alkaline protease isolated from culture filtrate of *B. subtilis* NCIM 2713 by ammonium sulphate precipitation had molecular weight 20 kDa (Mane and Bapat, 2001). However, a newly isolated *B. subtilis* PE-11 shown to have a relative low molecular weight of 15 kDa by SDS-PAGE (Adinarayana *et al*., 2003).

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