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Intergeneric Protoplast Fusion by Combining Genes to Improve Lipase and α-Amylase Enzyme Activities

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ABSTRACT

In order to produce new modified strains with improved lipase and α-amylase productivity for industrial and commercial use, the lipase gene (*lip3*) from *Pseudomonas aeruginosa*, *Pa* (Km^r Tc^r Sm^s/Rif^s Cm^r Nm^r), the α-amylase genes (*amyE* and *dltB*) from *Bacillus subtilis*, *Bs* (Km^s Tc^s Sm^r) and genes from *Bacillus thuringiensis*, *Bt* (Rif^r Cm^s Nm^s) were combined using protoplast fusion. The pattern of antibiotic resistance was used as a selectable marker to screen for fusant strains from these three parental strains. Six *Pa*::*Bs* fusants were selected on medium containing kanamycin, tetracycline and streptomycin and five *Pa*::*Bt* fusants were selected on medium containing rifampicin, chloramphenicol and neomycin. Each of the 11 new fusant strains combined the properties of their corresponding parental strains. PCR amplification of the parental *P. aeruginosa* strain revealed a 162-bp fragment that represented the *lip3* gene for lipase enzyme production. The other parental *B. thuringiensis* strain displayed a 1167-bp fragment that represented the *dltB* gene for α-amylase. The five fusants of these two parents contained the two fragments. PCR amplification of the two parental strains (*P. aeruginosa*, *B. subtilis*) showed the presence of the 162-bp fragment of the *lip3* gene in *P. aeruginosa* and the 1066-bp fragment of the *amyE* gene in *B. subtilis*. The six *Pa*::*Bs* fusant strains also contained the two genes, as revealed by the 162- and 1066-bp fragments. Lipase and α-amylase activities were estimated in the three parental strains and their 11 fusants; some fusants displayed higher activities of both enzymes than the three parental strains. SDS-PAGE analysis of the proteins confirmed that all 11 fusant strains acquired and expressed many specific protein bands from the three parental strains.

Keywords: α-amylase, *amyE*, *dltB* and *lip3* genes, *Bacillus thuringiensis* and *B. subtilis*, lipase, protoplast fusion, *Pseudomonas aeruginosa*, SDS-PAGE

INTRODUCTION

Combinatorial genomics (Manyak and Carlson 1999) is used to describe a direct, rapid and powerful set of manipulations which allows large regions of random genetic materials or entire genomes from donor microbes to be transferred and expressed in easily cultured host microbes. As a result of these combining genomics methods, the natural products present in a previously unexplored population of microbes can be potentially produced in fermentable organisms. Furthermore, these technologies can be used to produce novel chemical structures of natural products, through protoplast fusion, as a tool of protein engineering, to achieve strains with higher enzyme productivity (Manyak and Carlson 1999). Combining genomics using protoplast fusion has been reported between different *Bacillus* species for instance, between *Bacillus thuringiensis* (*Bt*) and *B. megaterium* (Mu *et al.* 1995), *B. subtilis* (*Bs*) and *B. licheniformis* (Iaroslavtseva *et al.* 1985) and *Bs* and *Klebsiella oxytoca* (Lihua *et al.* 1999). Protoplasts of *Bt israelensis* mutants were isolated and induced to fuse to each other. A comparison of δ-endotoxin concentration between *Bt* fusion and wild type (WT) strains showed that the *Bt* fusion contained 1.48 times more toxin than the WT (Yari *et al*. 2002). Protoplasts of two *B. megaterium* strains were fused and the colonies formed were found to be mixed populations of individual bacteria containing parental, recombinant and segregating genotypes (Fodor and Alfoldi 1976).

protoplast fusion allowed the transfer of a small plasmid pBC16 between *B. cereus* cells and resulted in the isolation of hybrid cells having acquired the tetracycline resistant (Tc^r) phenotype harboring pBC16 (Kovtunenko *et al.* 1985). Stable hybrids were obtained by protoplast fusion between *Bs* and *Zymomonas mobilis* and all the hybrids were able to hydrolyze starch and possessed ampicillin-resistant and Tc^r phenotypes (Gokhale and Deobagkar 1994). Nazari *et al.* (2005) obtained two UV-mutated strains of *Streptomyces griseoflavus*, which were selected based on their resistance to crystal violet and were used in intraspecific protoplast fusion. One of the resulting fusants had a 81.8% higher production rate than WT *S. griseoflavus*. A successful system employed intergeneric protoplast fusion between *Bt*, a strictly anaerobic Gram-negative rod, and *Pseudomonas fluorescens*, a facultative anaerobic Gram-positive, to express insecticidal toxin crystal protein genes (*cry*) (Ali and Attalah 2004).

Lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) are produced by a wide variety of living organisms. Most microbial lipases are secreted into the extracellular medium, which makes them easily recoverable for use in industrial applications. Of particular interest are lipases made by *Pseudomonas* species that have properties compatible with those used in household detergents, synthesis of pharmaceuticals or agrochemicals and processing of fats (Wirth *et al.* 1989). On the other hand, α-amylase (E.C. 3.2.1.1) randomly hydrolyzes α-1,4 glucosidic linkages in starch, glycogen and related polysaccharides yielding dextrins, oligosaccharides, maltose and D-glucose (Xiao *et al*. 2006). Bacteria belonging to the genus *Bacillus* have been widely used for the commercial production of thermostable α-amylase and industrial processes such as production of ethanol and high-fructose corn syrup, baking, in laundry washing pow-

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Table 1 Characteristics of specific primers for three genes used in the study.

Enzymes	Sequences of primers (5′–3′)	Genes	Bacterial sp.	Product size (bp)
α-amylase	F: TACAGCACCGTCGATCAAAA	*amyE*	*Bacillus subtilis*	1066
	r: ATTCGGGTTCGAGAGTTCCT			
	F: AGTGCGGCATGATAAAGACC	*dltB*	*B. thuringiensis*	1167
	r: TGACCGCATATGGATCATTT			
Lipase	F: AAACGATTCCTCCTCGGTCT	*lip3*	*Pseudomonas aeruginosa*	162
	r: AGGTAGGCGATCTCCAGGTT			

ders and dish-washing detergents, textile desizing, and paper recycling (Bano *et al.* 2009). Genes encoding enzymes, such as the lipase gene (*lip3*), were cloned from the *Pseudomonas aeruginosa* (*Pa*) strain LST-03 and expressed in *E. coli*. The cloned sequence includes an open reading frame (ORF) consisting of 945 nucleotides, encoding a protein of 315 amino acids (*lip3* lipase, 34.8 kDa) (Ogino *et al.* 2004). In addition, Liu *et al.* (2010) cloned the α-amylase *amyE* gene from *Bs* BF768 into vector pWB980 and over-expressed it in *Bs* WB600; secreted AmyE showed high activity (723 U/ml). Recombinant AmyE was purified to a specific activity of 36 U/ mg having optimal activity at pH 6.0 and 60°C.

The aim of this study was to produce new modified strains by combining lipase and α-amylase genes obtained from *Pa*, *Bt* and *Bs* by using intraspecific protoplast fusants to enhance lipase and α-amylase productivity for industrial and commercial use.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Pa was obtained from the American Type Collection (ATC) and both *Bs* and *Bt kurstaki* HD-1 were obtained from the *Bacillus* Genetic Stock Center (BGSC), Ohio State University. The three parental strains were grown in Luria-Bertani (LB) medium (pH 7.3) at 37°C with vigorous aeration (Davis *et al*. 1980).

Chemicals and growth medium

All chemicals and growth medium were purchased from Sigma-Aldrich Chemie GmbH, (Germany).

Protoplast formation

Protoplast (defined here as a modified, fused cell) formation, regeneration and fusion were performed according to Rajendran *et al.* (1994). The three bacterial strains – *Pa*, *Bt* and *Bs* – were incubated in Lb medium at 30°c for 24 h with shaking. The cells were harvested by centrifugation at 5000 rpm for 10 min and washed twice with protoplast buffer (10 mM Tris-HCl buffer, pH 7.2, containing 600 mM sucrose and 25 mM EDTA). lysozyme was added at a final concentration of 4 mg/ml to protoplast buffer and the cell suspension was incubated at 42°C for 3 h. Protoplast formation was confirmed by staining with methylene blue and then observed under a light microscope. The viability of protoplasts was determined by plating different dilutions of protoplasts in regenerating medium (Rajendran *et al.* 1994).

Protoplast regeneration and fusion

The protoplasts in the mixture were collected by centrifugation at 3000 rpm for 10 min. The precipitate was washed with Tris-HCl buffer then diluted and plated on LB medium to allow all protoplasts to regenerate. The colonies were counted after 48 h. one ml of mixed protoplast suspensions of *Pa* + *Bt* and *Pa* + *Bs* was added to 9 ml of 25% PEG 6000 and incubated at 30°c. Aliquots of 100 µl from each mixture were taken every 10 min and added to selective agar medium and overlaid on the same selective medium to screen for fusants.

Design of specific primers for lipase and α-amylase genes

The forward and reverse primers for *amyE* and *dltB* genes, the structural genes encoding α-amylase production, were designed from GenBank (FJ463162 and AE017355, respectively), and for the *lip3* gene, coding for lipase production, from AB125368, as shown in **Table 1**.

DNA extraction and PCR amplification of lipase and α-amylase genes

DNA was extracted from the parental strains and their new fusants following the method of Ben-Dov (1999). Genomic DNA was prepared from an exponential overnight phase in LB medium (Davis *et al*. 1980). Aliquots of 10 ml of bacterial culture were harvested by centrifugation at 12,000 rpm for 15 min and washed once in sterile distilled water. the pellets were resuspended in 400 µl of lysis buffer containing 2% glucose, 50 mM Tris-HCl (pH 8.0), 25 mM EDTA, 3 mg/ml lysozyme and 200 mg/ml RNase. The cell suspension was incubated for 1 h at 37°C. Further DNA extraction was performed as described by Sambrook *et al.* (1989).

Amplification was performed according to Pinto and Fiuza (2003) in a GeneAmp 9600 Perkin Elmer (Martinsburg, West Virginia, USA) thermal cycler in a total volume of 25 µl containing 50 ng DNA, 1 mM of each primer, 200 mM dNTP, 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Promega, Madison, USA). PCR was performed under the following conditions: 5 min at 95°C and then 40 cycles of 30 sec at 94°C, 1 min at 52°C and 1 min at 72°C and a final extension step at 72°C for 5 min. The PCR products were separated in a 1.2% agarose gel containing 0.2 mg/ml of ethidium bromide and were visualized using a Gel Doc XR System (Bio-Rad Laboratories, Inc., Cali, USA).

Antimicrobial susceptibility

Seven antibiotics were used with final concentrations as follows: rifampicin (Rif) 100 µg/ml, ampicillin (Amp) 100 µg/ml, streptomycin (Sm) 200 µg/ml, kanamycin (km) 40 µg/ml, tetracycline (Tc) 15 µg/ml, chloramphenicol (cm) 35 µg/ml and neomycin (Nm) 40 µg/ml. the Kirby-Bauer disc diffusion method for antimicrobial susceptibility test was used (NCCLS 1992).

SDS-PAGE analysis of proteins

The parental and fusant strains were grown in separate suspensions following the method of Von Tersch and Gonzalez (1994). Nutrient broth (100 ml) was inoculated into 500-ml flasks with one loop of bacteria and shaken for 3 days at 220 rpm at 30°C. The suspension was centrifuged for 10 min at 10,000 rpm at 4°C. The pellet was washed twice with high salt TNT-1 buffer (50 mM Tris HCl pH 7.5, 1.0 M NaCl and 0.05% Triton X-100) followed by two washes with TNT-2 buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl and 0.05% Triton X-100). One ml of aliquot was solubilized by heating in Laemmli buffer (10% (w/w) glycerol, 5% (w/w) β-mercaptoethanol, 1% (w/v) SDS, 0.188 M Tris-HCl pH 6.8 and 0.01% (v/v) bromphenol blue) at 100°C for 5 min. The aliquot was centrifuged at 13,000 rpm for 5 min and the supernatants containing solubilized proteins were fractionated by size using 15% SDS-PAGE according to Laemmli (1970) to compare the products secreted by the parental strains and those secreted by the new fused strains. After size fractionation, the proteins were visualized by staining with Coomassie Blue R-250 dye.

Table 2 Antibiotics resistant patterns of the three parental strains and their fusants.

Parental strains and their fusants	Fusant no.	Antibiotics resistant patterns								
		Km	Tc	Rif	Sm	Amp	Cm	Nm	Km Tc Sm	Rif Cm Nm
P. aeruginosa (*Pa*)		+	+	-	-	+	+	+	-	-
B. subtilis (*Bs*)		-	-	-	+	+	+	+	-	-
Pa :: *Bs*	1								+	
	2								+	
	3								+	
	4								+	
	5								+	
	6								+	
P. aeruginosa (*Pa*)		+	+	-	-	+	+	+	-	-
B. thuringiensis (*Bt*)		+	+	+	-	+	-	-	-	-
Pa :: *Bt*	1									+
	2									+
	3									+
	4									+
	5									+

RESULTS

Antimicrobial susceptibility

Antibiotic resistance of the three parental strains was examined on seven different LB agar plates, each of which was supplemented with one of the antibiotics; Rif, Amp, Sm, Km, Tc, Cm and Nm at 100, 100, 200, 40, 15, 35 and 40 µg/ml, respectively. The results, summarized in **Table 2**, indicate that the three parental strains were Amp resistant (Amp^r). both *Pa* and *Bs* were similarly resistant to Cm and Nm (Cm^r and Nm^r) while *Bt* was sensitive to them (Cm^s and Nm^s). Moreover, *Pa* and *Bt* were resistant to Km and Tc (Km^r and Tc^r), while *Bs* was sensitive to them (Km^s and Tc^s). Regarding the remaining antibiotics, Rif and Sm, *Bt* was resistant to Rif (Rif^r) and *Bs* to Sm (Sm^r) only. The antibiotic resistance patterns of the parental strains were used as selectable markers to screen for the fusant strains. Six fusants resulting from *Pa*::*Bs* protoplast fusion were selected on medium containing km, Tc and Sm and five *Pa*::*Bt* fusants were selected on medium containing Rif, cm and Nm (**Table 2**). Each of the 11 new fusant strains combined the antibiotic properties of their corresponding parental strains (**Table 2**).

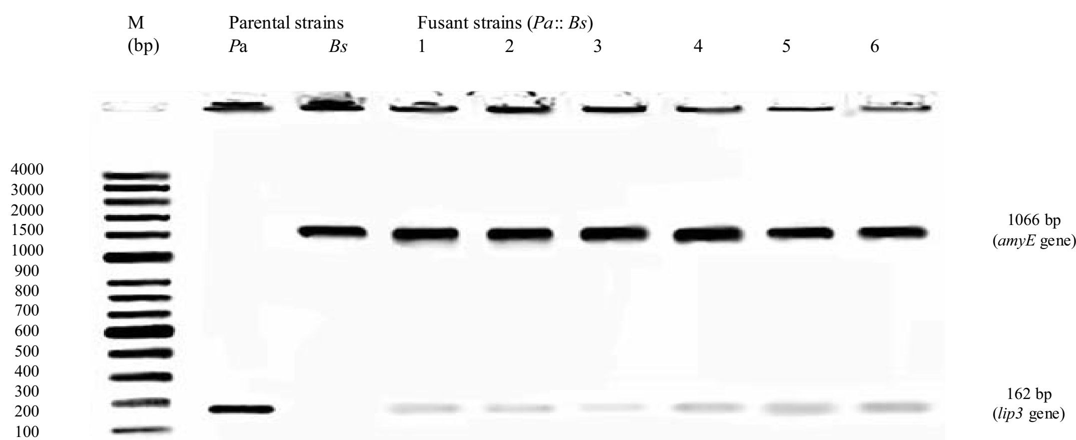

Fig. 1 PCR amplifications of the two parental strains, *P. aeruginosa* (*Pa*), *B. subtilis* (*Bs*) and their six fusant strains.

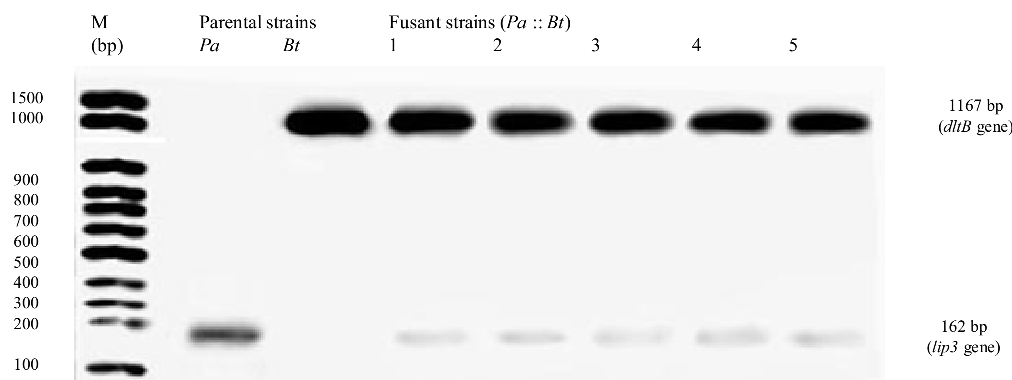

Fig. 2 PCR amplifications of the two parental strains, *P. aeruginosa* (*Pa*), *B. thuringiensis* (*Bt*) and their five fusant strains.

Detection of parental lipase and α-amylase genes in the fusant strains

PCR amplification of *Pa* parental strain revealed the 162-bp fragment that represented the *lip3* gene for lipase enzyme production. On other hand the other two parents' strains, *Bt* and *Bs* displayed two fragments (1167 and 1066 bp) that represented the *dltB* and *amyE* genes for α-amylase enzyme production (**Figs. 1, 2**).

The six fusant strains resulting from *Pa*::*Bs* protoplast fusion revealed the fragments of the two genes, i.e. 162 and 1066 bp (**Fig. 1**), while the other five fusant strains resulting from *Pa*::*Bt* protoplast fusion revealed the two parental fragments with the expected sizes (1167 and 162 bp), as shown in **Fig. 2**.

In the present study, gene transfer was evident with the lipase gene (*lip3*) and α-amylase genes (*amyE* and *dltB*) in the fusants and genetic modifications occurred after protoplast fusion.

Expression of the parental protein bands in the fusant strains

The SDS-PAGE protein banding patterns of the three parental strains and their 11 fusants are presented in **Fig. 3**. SDS-PAGE analysis of total proteins of the two parental strains (*Pa* and *Bs*) revealed a total of 31 and 21 protein bands, respectively with molecular weights (MW) ranging from 184 to 5 kDa (**Table 3**). In contrast, the six *Pa*::*Bs* fusants showed variable numbers of bands ranging from 16 in fusant no. 3 to 28 in fusant no. 1. The number of *Bs* protein bands expressed in *Pa*::*Bs* varied from a high of 18 bands in fusant no. 1 to a low of 8 bands in fusant no. 3, while 13 bands were shown in fusant no. 4, 12 bands in fusant no. 6 and 11 bands in the two remaining fusants. Moreover, the number of *Pa* protein bands expressed in *Pa*::*Bs* fusants also varied from 14 in fusants no. 4 and 6 to 8 in fusant no. 3 (**Fig. 3; Table 3**).

The six fusants were genetically characterized based on their absence and presence of the expressed *Bs* protein bands, where four protein bands with MWs of 95, 75, 59 and 18 kDa were expressed in the six fusants; two others (38 and 5 kDa) were expressed in five fusants only and disappeared in fusant no. 3. Two bands (159 and 139 kDa) appeared in four fusants and disappeared in fusants no. 2 and 3 and the 7-kDa band disappeared in fusants no. 3 and 6. Six bands (184, 180, 51, 24, 22 and 19 kDa) appeared in three fusants and disappeared in the other three. Each of the three remaining protein bands (117, 27 and 12 kDa) and three other bands (30, 13 and 10 kDa) appeared only in one fusant. The six fusants were characterized according to the expressed of the *Pa* protein bands in them. One band (103 kDa) existed in the six fusants, while the other expressed bands varied as follows: two bands (86 and 31 kDa) existed in five fusants, two others (161 and 35 kDa) in four fusants but these disappeared in fusants no. 1 and 2, 10 bands appeared in three different fusants, 6 bands in two fusants and five bandsin one fusant.

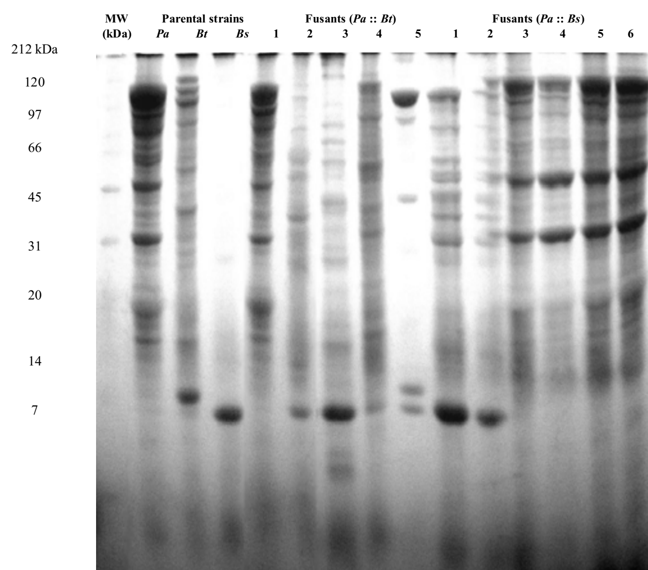

Fig. 3 SDS-PAGE protein profiles of the three parental strains and their fusant strains resulted from protoplast fusion. Protein marker purchased from Biomatik (Wilmington, USA) with 9 molecular weight bands (kDa) is presented in the far-left lane.

In addition, SDS-PAGE analysis of the total proteins of the two parental strains (*Pa* and *Bt*) revealed a total of 31 and 23 protein bands, respectively (**Table 3**) while the five *Pa*::*Bt* fusants showed a variable number of bands ranging from 29 bands in fusant no. 1 to 35 bands in fusant no. 5.

The number of *Bt* protein bands expressed in *Pa*::*Bt* varied from a high of 17 and 16 bands in fusants no. 2 and 5, respectively to a low of 10 bands in fusant no. 1, while the other two fusants showed 14 bands in fusant no. 4 and 13 bands in fusant no. 3.

Moreover, the number of *Pa* protein bands expressed in *Pa*::*Bt* fusants also varied from 19 in fusants no. 1, 4 and 5 to 17 bands in fusants no. 2 and 3. The five fusants were discriminated based on the absent or presence of the expressed *Bt* protein bands whereas five bands (150, 27, 24, 22 and 19 kDa) were expressed in five fusants, six other bands (180, 121, 95, 81, 7 and 5 kDa) were expressed in four fusants but disappeared in only fusant no. 1. Three bands (184, 159, 75 and 13 kDa) were expressed in three fusants while three other bands (48, 32 and 18 kDa) appeared in two fusants. The remaining three protein bands appeared in one fusant. Low MW bands (12 and 59 kDa) expressed only in fusant no. 1 and the 63-kDa bands in fusant no. 2.

Similarly, the five fusants could be characterized according to the expressed of *Pa* protein bands in them.

DISCUSSION

In Gram-positive bacteria, *Bacillus subtilis* and related bacteria, signal peptide-bearing secretory proteins are translocated through the cytoplasmic membrane and fold into their native conformation on the outside of the cell. The products of the *Bs amyE* and *dltB* genes separately influence post-translocational stages of the secretion process by mediating proteolytic degradation and by folding secretory proteins. Inactivation of either *amyE* or *dltB* in *Bs* increases the yield of secretory proteins released into the culture medium in an intact and biologically active conformation. Stephenson *et al.* (2002) studied the combined influence of *wprA* and *dltB* genes and they constructed a *wprA*/*dltB* double mutant and found that it did not have an additive effect on secretion and caused a significant reduction in the yield of α-amylase. The activities of the *wprA* gene and the *dlt* operon interact in a negative way to influence the growth cycle and protein secretion.

An antibiotic resistance pattern was used as selectable markers by Belykh *et al.* (1983), who used medium containing Rif and Tc to select recombinants of the fused protoplasts of *Bt* var. *galleriae* and *B. cereus* that carrying the plasmid pBC16 responsible for resistance to Tc. In the same manner, fusion of *B. cereus* protoplasts resulted in isolation of hybrid cells having acquired the Tc^r phenotype (harboring pBC16) with high frequencies 10^{-2} to 10^{-3} (Kovtunenko *et al.* 1985).

The *amyE* gene from *Bacillus licheniformis*, which is widely used for the industrial hydrolysis of starch, was mutated (*amyEM*), then amplified by PCR and inserted into pBV220 and pPIC9k to obtain the recombinant vector pBV220-*amyEM* and pPIC9k-*amyEM*, respectively (Du *et al.* 2006). These recombinant vectors were transformed into corresponding competent *E. coli* DH5-α and *P. pastoris* GS115 cells, respectively. The resulting recombinant strains, DH5-α/pBV220-*amyEM* and GS115/pPIC9k-*amyEM*, were then screened by measuring the enzymatic activity and

Table 3 SDS-PAGE analysis of total proteins of the three parental strains and their fusants.

* Band No.	MW (kDa)	Parental strain		Fusants *Pa* :: *Bt*					Parental strain	Fusants *Pa* :: *Bs*					
		Pa	*Bt*	1	2	3	4	5	*Bs*	1	2	3	4	5	6
1	184		◆		◆			◆							
2	180		◆		◆	◆	◆	◆							
3	170	+		+	+	+	+	+			+			+	+
4	161	+		+	+	+	+	+				+	+	+	+
5	159		◆	◆	◆			◆							
6	156	+					+				+				
7	150		◆	◆	◆	◆	◆	◆							
8	148	+									+	+			
9	139														
10	136	+		+			+								
11	131	+			+			+		+		+			+
12	121		◆		◆	◆	◆	◆							
13	117														
14	112	+		+	+	+	+	+							
15	110	+		+	+	+	+	+					+	+	+
16	103	+		+		+				+	+	+	+	+	+
17	95		◆		◆	◆	◆	◆							
18	86	+								+	+		+	+	+
19	81		◆		◆	◆	◆	◆							
20	76	+		+	+	+	+						+	+	
21	75		◆		◆	◆	◆								
22	68	+		+	+	+	+	+		+	+				
23	67	+											+	+	+
24	63		◆		◆										
25	60	+				+		+							
26	59		◆	◆											
27	55	+			+	+	+	+							
28	51														
29	49	+													+
30	48		◆			◆		◆							
31	42	+		+	+		+			+			+	+	
32	40	+		+	+	+	+	+		+		+			
33	38														
34	35	+				+	+	+				+	+	+	+
35	34	+								+	+		+		
36	32		◆	◆				◆							
37	31	+		+	+						+	+	+	+	+
38	30		◆												
39	29	+		+		+		+		+			+		
40	27		◆	◆	◆	◆	◆	◆							
41	25	+		+	+	+	+	+				+			
42	24		◆	◆	◆	◆	◆	◆							
43	23	+		+	+	+	+	+							
44	22		◆	◆	◆	◆	◆	◆							
45	21	+		+	+	+	+	+		+	+				+
46	20	+		+	+	+	+	+					+	+	+
47	19		◆	◆	◆	◆	◆	◆							
48	18		◆		◆		◆								
49	16	+					+				+		+		
50	13		◆	◆	◆			◆							
51	12		◆	◆											
52	11	+		+	+	+	+	+						+	
53	10		◆												
54	9	+		+				+			+		+		+
55	8	+		+	+			+							
56	7		◆		◆	◆	◆	◆							
57	6	+					+	+		+	+				+
58	5		◆		◆	◆	◆	◆							
Total bands		31	23	29	34	30	33	35	21	28	23	16	27	23	26
*				10	17	13	14	16	#	18	11	8	13	11	12
**				19	17	17	19	19	##	10	12	8	14	12	14

Existence of protein bands: in *P. aeruginosa* (+), *B. thuringiensis* (◆) and *B. subtilis* ( )
* = Numbers of *Bt* bands expressed in *Pa* :: *Bt*, ** = *Pa* bands expressed in *Pa* :: *Bt*
\# = Numbers of *Bs* bands expressed in *Pa* :: *Bs*, ## = *Pa* bands expressed in *Pa* :: *Bs*

SDS-PAGE. DH5-α/pBV220-*amyEM* were induced by temperature and GS115/pPIC9k-*amyEM* by methanol. In contrast to the parent cells, α-amylases were expressed in both recombinant strains. In *E. coli*, the MW was approximately 55 kDa and the optimal temperature and pH of the recombinant α-amylase were 80-90°C and 6.0, respectively. The recombinant amylase had high activity at pH 5.0-5.5 compared to the wild type. In *Pichia pastoris*, the recombinant amylase was secreted in the medium and the MW was 60 kDa for the putative post-translational modifications and the

optimal pH shifted to 5.5. The specific activity of α-amylase produced by *P. pastoris* was 102 U/mg. This result indicates that α-amylase was secreted into the culture medium with high efficiency in the recombinant *P. pastoris*. High activity at higher temperature and lower pH provides the recombinant amylase potential applications in industry. Although medium-temperature α-amylase (AmyE) has some practical advantages, its low yield limits its applications.

The MWs of lipase and α-amylase protein bands in the three parental strains and their fusants (**Table 3**) were similar to the MWs obtained from several reports. For instance, protein analysis of α-amylase bands showed two bands (95 and 75 kDa) in *Bs* and some of their *Pa*::*Bs* fusants, confirming the results of Tsukamoto *et al.* (1988), who reported five kinds of α-amylase exhibiting starch hydrolyzing activity with approximately 110, 95, 85, 75, and 60 kDa. The α-amylases were 518 amino acids in length with an estimated MW of 59 kDa; the first 33 amino acids act as the signal peptide that is involved in the secretion of the exported protein. The extracellular form would therefore have 485 amino acids with an estimated MW of 55,372 kDa (Tsukamoto *et al.* 1988). Such a reported α-amylase band with 59 kDa existed in *Bs* and some of its *Pa*::*Bs* fusants (**Table 3**). Moreover, *Pa* lipase was composed of a single subunit (29 kDa, pI 4.9), which was capable of a variable degree of aggregation and which exhibited lipase activity (Gilbert *et al.* 1991). The 29-kDa lipase band was clearly detected in the parental *Pa* strain and in some of their fusants (**Table 3**). Two other extracellular lipases with 16 and 55 kDa were detected in *Pa* strain and its fusants (**Table 3**), which was confirmed by Saeed *et al.* (2005), who isolated and purified two different extracellular lipases with MW = 15.5 and 54.97 KDa from *Pa* Ps-x.

Generally, 11 new fusant strains with their specific properties were obtained and selected from the regenerated colonies. the results show that these fusants exhibited the desirable characters of both the parental strains, either in their existing three genes (*amyE*, *dltB* and *lip3*) or in the protein bands. The results are supported by the findings of many reports. For instance, Mu *et al.* (1995) used 40% PEG and obtained four stable protoplast fusants with both pesticide ability and decomposing phosphate activity which were transferred to them from the two parental strains *Bt kurstaki* and *B. megaterium* var. *phosphaticum*, respectively. Fodor and Alfoldi (1976) analyzed the fused colonies of protoplasts of two *B. megaterium* strains and they found mixed populations of individual bacteria apparently containing parental, recombinant, and segregating genotypes. Moreover, recombinants between *Bs* and *B. licheniformis* were prepared by fusion of the bacterial protoplasts; all the hybrids acquired the specific properties of *Bs*. their formation was based on the whole chromosome of *Bs* and recombination of separate fragments of *B. licheniformis* with it. Iaroslavtseva *et al.* (1985) concluded that, contrary to transformation of isolated DNA, protoplast fusion may result in formation of interspecies recombinants with respect to different operons of amino acid synthesis (Iaroslavtseva *et al.* 1985). In the present study, the possibility of obtaining interspecific bacterial hybrids by PEG-assisted fusion of protoplasts from *Bt* and *Pa* has been genetically examined. genetic data confirms, with great probability, that cytological fusion takes place. Moreover, the protoplast fusion method resulted in stable recombinants, which was also observed by Tsenin *et al.* (1983) and Fischer *et al.* (1984) in *B. thuringiensis*.

Consequently, a successful intergeneric protoplast fusion was performed between Gram-negative *P. aeruginosa* harboring the lipase *lip3* gene with each of the two Gram-positive strains, *B. subtilis* and *B. thuringiensis*, that harboring α-amylase *amyE* and *dltB* genes, respectively, to construct new enhanced hybrid strains harboring all of the lipase and α-amylase genes, and their combined properties.

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