

Poly(hydroxyalkanoate) Production by *Cupriavidus necator* from Fatty Waste Can Be Enhanced by *phaZ1* Inactivation

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PHA production from waste oils or fats requires microorganisms that should be both excellent PHA producers and equipped with enzymatic activities allowing hydrolysis of triglycerides. Unfortunately, microbes with the combination of substrate-utilization and PHA production are not currently available, and the strategies to be adopted are the use of costly commercial enzymes, or genetic modification of microorganisms exhibiting high PHA product yields.

In the present work, after a general investigation on the ability of *Cupriavidus necator* to grow on a number of fatty substrates, the possibility to enhance PHA production by limiting intracellular depolymerisation, was investigated. By knocking out the related *phaZ1* gene, the construction of *C. necator* recombinant strains impaired in depolymerase (PhaZ1) activity was achieved. The polymer yield of the recombinant strain was finally compared to that of the parental *C. necator* DSM 545.

Key words:

poly(hydroxyalkanoates), glycerol, lipase activity, depolymerase, *Cupriavidus necator*

Introduction

Although PHAs are regarded as an effective substitute for conventional plastics for a number of medical and agricultural applications¹ and food packaging², their full-scale manufacturing is hampered by high production costs³. Factors affecting the cost of PHAs include raw materials availability, suitable process design, and downstream processing^{4,5}. Since almost 50 % of the total production costs can be attributed to the carbon source for microbial growth and polymer production, the selection of renewable, cheap carbon feedstock, specially generated from industrial or agricultural by-products, can provide a way to reduce the price^{6,7,8}. To that end, different industrial by-products, such as whey^{9,10}, molasses¹¹, starch, and waste oils and glycerol, have been investigated as start materials for PHA production^{12,13,14,15}. In this perspective, fatty residues from slaughterhouse represent a promising raw material. For Europe, the amount of animal lipids, also causing expensive disposal problems, is estimated a half a million tons per year¹⁶.

However, PHA production from waste oils or fats requires microorganisms that should be both excellent PHA producers as well as equipped with enzymatic activities allowing hydrolysis of triglycerides.

Cupriavidus necator (formerly *Ralstonia eutropha*) is one of the best known bacteria among PHA-producing microorganisms^{15,17}. The production of different kinds of PHAs by *C. necator* using several inexpensive feedstock, including glycerol, has been recently reported^{18,19,20,21,22}, indicating that some strains of this bacterial species could be used for the conversion of fatty residues into PHA. As an alternative, the use of costly commercial enzymes or genetic modification of microorganisms exhibiting high PHA product yields would be required²³. In the case of *C. necator*, its lipase activities could most likely be improved by physiological/technological studies in terms of biomass production and PHA content, but the help of commercial enzymes may still be necessary. A possible strategy to help increase the final PHA yield in a bacterial strain already possessing both high polymer production ability and acceptable triglyceride hydrolytic activity, could originate from the relevant findings regarding *C. necator* PHA metabolism, physiology,

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and biochemistry, reported over the past few decades^{24,25,26}. These studies are including poly(3-hydroxybutyrate) depolymerase enzymes, known as PhaZ, and their role in PHB utilization and intracellular granule formation. The main depolymerase, PhaZ1, was reported to play a significant role in PHB mobilization²⁶. The active role of these enzymes in PHB consumption has been shown earlier by analysing PHB content in *phaZ1* and *phaZ2* deletion strains²⁶, and more recently, further elucidations of their role in *C. necator* have been reported^{27,28}.

Several studies have indicated that PHB synthesis and PHB degradation can happen simultaneously in *Ralstonia eutropha*^{29,30,31} and that there is constitutive expression of PHB synthase and PHB depolymerase³². Therefore, limiting intracellular depolymerisation could lead to enhancement of the final amount of polymer accumulation. In the present work, by knocking out the relative depolymerase gene, the construction of a *C. necator* recombinant strain impaired in PhaZ1 was achieved.

The polymer yield of the recombinant strain, and especially its ability to maintain as much polymer as possible at the end of the accumulation phase and possibly during the downstream process, was finally compared to that of the parental *C. necator* DSM 545.

Materials and methods

All chemicals and media components were of analytical grade standard. Fatty carbon sources were of commercial origin (corn oil and lard), while udder and tallow were kindly provided by Prof. Mittelbach (Karl-Franz University of Graz-Austria).

Culture media were autoclaved at 120 °C for 20 minutes before utilization. When necessary, liquid media were solidified adding 1.5 % agar-agar.

Bacterial strains, plasmids, and growth conditions

The strains of *Cupriavidus necator* and *Escherichia coli* as well as plasmids used in this study are listed in Table 1.

C. necator strains were cultured at 30 °C in minimal salt medium DSMZ 81 (Deutsche Sammlung Mikroorganismen, <http://www.dsmz.de/>, Germany) with glucose as carbon source. *E. coli* strains were cultured at 37 °C in Luria-Bertani medium (LB) containing (g L⁻¹): yeast extract 5, tryptone 10, and NaCl 10. Antibiotics were supplemented as required at the following concentrations: for *C. necator* kanamycin 100 µg mL⁻¹, gentamicin 40 µg mL⁻¹, tetracycline 8 µg mL⁻¹; for *E. coli* tetracycline 10

Table 1 – Bacterial strains and plasmids used in this work

Strain or plasmid	Genotype and sequences	Source or reference
Strains		
<i>Cupriavidus necator</i> DSM 545	Wild-type	42
<i>Cupriavidus necator</i> sp-1	<i>phaZ1::Km^r</i>	This work
<i>Escherichia coli</i> JM109	<i>recA, supE, supF, lacYI</i>	43
<i>E. coli</i> XL1-Blue	<i>recA1 lac [F' proAB lacF^r ZAM15 Tn10 (Tet^r)] thi</i>	38
Plasmids		
pSUP102	Cm ^r , Tc ^r , harboring <i>mob</i> site	44
pSUP102- <i>phaZ1</i>	Tc ^r , Mob ⁺ , a <i>phaZ1</i> fragment of <i>C. necator</i> subcloned in pSUP102	45
pSUP102- <i>phaZ1::Km^r</i>	Tc ^r , Mob ⁺ , a <i>phaZ1</i> fragment of <i>C. necator</i> subcloned in pSUP102 interrupted by the <i>Km^r</i> from pHM2	This work
pHM2	Broad host range plasmid Tc ^r , Mob ⁺ , <i>Km^r</i>	41

µg mL⁻¹, kanamycin 50 µg mL⁻¹, ampicillin 100 µg mL⁻¹. Liquid cultures were shaken at 150 rpm.

Cultivation procedure and PHA production

For the evaluation of bacterial growth and PHA production, a two-step cultivation procedure was carried out, as previously described³³. In the case of recombinant *C. necator* strain, 100 µg mL⁻¹ kanamycin were added.

In the first step, aimed to produce biomass, the microorganisms were grown under shaking at 150 rpm, in aerobic conditions at 30 °C, in 500 mL flasks containing 100 mL of DSMZ 81 medium with 20 g L⁻¹ glucose, using a 0.25 % (vol/vol) overnight pre-culture (OD_{600 nm} = 1.5) as inoculum. After 24 h, the cells were harvested by centrifugation at 4000 g at 4 °C for 15 minutes. In the second step, to promote PHA synthesis, the cells were transferred into a 100 mL nitrogen-free DSMZ 81 medium (pH 7.0) for 2–3 days in shaking flasks at 150 rpm; the medium was supplemented with 20 g L⁻¹ of one of the following carbon sources: corn oil, glycerol, myristic acid, stearic acid, palmitic acid, oleic acid, bacon rind, udder, tallow. All the experiments were performed in triplicate, and after incubation the cells were harvested by centrifugation, and the pellets stored at –20 °C before chemical analysis.

Analytical procedures

For cell dry mass determination, 5 mL of culture broth was centrifuged in pre-weighed tubes, the

remaining biomass pellet was frozen and lyophilised, and gravimetric difference against empty tubes was calculated.

PHA concentration was determined in centrifuged cells by the method of Braunegg *et al.*³⁴ 3-hydroxyalkyl esters were quantified by gas chromatography (GC) with a silica fused capillary column AT-WAX (Alltech Italia s.r.l., Milan, Italy) and a flame ionization detector. The gas carrier was helium, the injection port temperature was 250 °C, the detector temperature 270 °C, and the oven temperature 150 °C. The GC-temperature programme was as follows: initial oven 90 °C (maintained for 1 min), with increases of 5 °C per minute to a final temperature of 150 °C (maintained for 6 min). The internal standard was benzoic acid, and the external standards were: 3-hydroxybutyric acid (Sigma-Aldrich, Italy) and P(3HB-co-3HV) copolymer (Biopol TM; Imperial Chemical Industries, Great Britain). Results were expressed as percentage of PHA on the bacterial biomass.

PHA monomer composition was determined by nuclear magnetic resonance (¹HNMR), as previously described³⁵, and the spectra registered on a Varian Gemini 200 (200 MHz) spectrometer interfaced with a Sparc4(Sun) console and software VNMR6.1B. The co-monomers compositions in the co-polymers were calculated by using the ratio between the normalized integrals of CH₃ signals of 3-hydroxyvalerate and the normalized integrals of CH₃ signal of 3-hydroxybutyrate. The presence of 3-hydroxybutyrate was revealed by the peaks at 5.25 ppm, 2.55 ppm and 1.30 ppm, whose integrals and multiplicity were ascribed respectively to its CH, CH₂ and CH₃ proton signals. The presence of 3-hydroxyvalerate in the copolymer was revealed by the peaks at 0.95 ppm and 1.65 ppm ascribed respectively to its CH₂ and CH₃ proton signals.

Titrimetric assay was as previously described³⁶. In short, 5 mL of the supernatant was added to 50 mL of 5 % (v/v) corn oil emulsion in 50 mmol L⁻¹ Tris-HCl buffer (pH 8), containing 5 % (w/v) of arabic gum. The mixture was incubated at 37 °C for 3 h and, every 30 minutes, 5 mL were sampled and added to 10 mL ethanol to stop the reaction. The released fatty acids were titrated with 0.05 mol L⁻¹ NaOH using phenolphthalein as an indicator. The difference in titer values between samples and blank was used to calculate the amount of released fatty acid. One unit of lipase was defined as the amount of enzyme that released 1 μmol of fatty acid per minute under assay conditions.

DNA isolation and manipulation

Plasmid and total DNA isolation, agarose gel electrophoresis and transformation of *E. coli* strains

were carried out as described³⁷. All DNA-manipulating enzymes were used by following the instructions of the manufacturers.

Construction of recombinant *C. necator* strain

For *phaZ1* disruption, SP-L and SP-R (5'-CATCAAGCTGCTCAAGGATG-3' and 5'-AAGAGATCTACCCGCAGCTG-3') primers were designed on the sequence of *phaZ1* gene, amplifying a region of 918 bp (from nucleotide 302 to 1194 of the gene). The amplified fragment was cloned into the pDrive plasmid, giving rise to plasmid pDRPT. A *Bam*HI-*Hind*III fragment containing the amplified *phaZ1* fragment was isolated from plasmid pDRPT and cloned between the *Bam*HI-*Hind*III sites of the suicide vector pSUP102 (Cm^r, Tc^r)³⁸ resulting in plasmid pSUP102-*phaZ1*. The amplified fragment of *phaZ1* gene, inside pSUP102-*phaZ1*, was interrupted by a kanamycin resistance gene (*Km*^r) introduced into the unique *psfI* site present in the construct pSUP102-*phaZ1*. The resulting plasmid was named pSUP102-*phaZ1*::*Km*. The recombinant plasmid pSUP102-*phaZ1*::*Km* was transferred from the donor strain *E. coli* JM109 to the recipient *C. necator* through a three-parental mating. Conjugation between *E. coli* strains and *C. necator* was performed on solidified nutrient broth medium (NB: meat extract 1g L⁻¹, yeast extract 2 g L⁻¹, peptone 5 g L⁻¹, NaCl 5 g L⁻¹). After 24 h incubation at 30 °C, the cells were resuspended in saline solution (0.9 g NaCl L⁻¹) and dilutions plated on selective media DSMZ81 containing glucose as carbon source with addition of kanamycin (100 μg mL⁻¹). Colonies of *C. necator* able to grow in the presence of *Km* and sensitive to *Cm* were selected.

Results and discussion

Among several bacterial species previously tested, *C. necator* was chosen as a model organism because it is well known as a proficient PHA producer, and because the complete genome sequence of a type strain revealed the presence of lipase genes³⁹.

Production of poly(hydroxyalkanoates) by *C. necator* DSM 545 from different carbon sources

Several preliminary tests were performed both in solid and liquid media on *C. necator* DSM 545 for its ability to metabolize triglycerides. The strain was found to be able to grow on a number of fatty substrates and, before approaching more complex fats as carbon source, a more accurate growth and PHA production test was assessed with corn oil, due to its emulsion easiness. With corn oil as substrate,

the content of extracellular lipase units in the supernatant reached 0.47 U mL^{-1} after 72 h growth, as determined by the titrimetric assay previously described⁴⁰.

Following this result, other fatty substrates with different complexity were tested, such as glycerol, some methylesters derived from different fatty acids (myristic, palmitic and stearic methyl esters) and oleic acid, up to directly using animal fatty waste (bacon rind, udder, tallow). All these substrates supported, at different rates, the growth of *C. necator*, and in all the samples the presence of PHA was revealed (Table 2). Especially glycerol, previously reported as a suitable substrate for growth and PHA production by *C. necator*¹⁹, was confirmed as the most suitable carbon source and therefore was used as a model for the subsequent experiments. Indeed, in a 2-step fermentation process, performed in flask as described above, from this structurally unrelated carbon source the strain synthesised interesting amounts of PHAs as revealed by GC analysis. ¹HNMR examination confirmed the presence of the P(3HB-co-3HV) copolymer, and provided the monomer composition (Table 2). ¹HNMR analysis indicated that most of the PHAs produced by the microbial strains were copolymers constituted predominantly by 3-hydroxybutyrate as major component, and 3-hydroxyvalerate as minor component.

Table 2 – Growth and PHA accumulation by *C. necator* DSM 545 in DSMZ81 medium containing different carbon sources. +: presence of PHA; nd: not determined. The data represent the mean of three replicates and standard deviation was always below 5 %.

Carbon source	Growth time (h)	CDM (g L ⁻¹)	% PHA on CDM	Monomer units (mol %)
Glycerol	72	6.35	73.27	P(3HB-co-1.5 % 3HV)
Waste Glycerol	72	0.95	+	P(3HB-co-2 % 3HV)
Waste Glycerol + 2.5 % v/v biodiesel	72	1.05	+	P(3HB-co-3 % 3HV)
Waste oil	96	0.93	+	nd
Oleic acid	96	1.60	+	nd
Myristic methyl ester	96	1.43	+	nd
Palmitic methyl ester	96	1.97	+	nd
Stearic methyl ester	96	2.11	+	nd
Saturated fraction (1 % w/v)	96	0.64	+	nd
Bacon rind	96	0.11	+	nd
Udder	96	2.26	+	nd
Tallow	96	0.93	+	nd

When waste glycerol was used, although the same co-polymer was formed, the growth was considerably limited, suggesting the presence of some unwanted compound in this waste residue. The supplement of 2.5 % v/v of biodiesel as co-substrate seemed to help the strain to grow.

Encouraging growth was also obtained by the direct use of animal fatty waste. Particularly interesting was udder, the fat extracted from the mammary glands of cow, giving appreciable growth rates. On the other hand, tallow, the rendered form of beef or mutton fat, and bacon rind only supported limited growth rates.

These findings confirmed that *C. necator* can express its own lipases for the conversion of fatty material into PHA, even if the efficiency was found to be different depending upon the substrate. Therefore, in order to possibly improve polymer production, irrespective of the carbon source used, a depolymerase approach was pursued.

Disruption of *phaZ1* gene in *Cupriavidus necator* with a cartridge containing a *Km^r*-cassette

In order to reduce intracellular PHA degradation, one of the intracellular PHA-depolymerases was inactivated by chromosomal integration of a cassette containing a kanamycin resistance gene (*Km^r*). In a previous work, part of the *phaZ1* depolymerase gene was cloned in the suicide plasmid pSUP102, thus obtaining plasmid pSUP102-*phaZ1*⁴¹. The gene *phaZ1* was interrupted in the unique *pstI* site by the *Km^r* gene. This construct was then transferred from *E. coli* to *C. necator* DSM 545 by conjugation. After conjugation, where a double crossing-over occurred and the *phaZ1* gene was replaced, some colonies of *C. necator* became Cm-sensitive and resistant to kanamycin. The 16S rDNA sequences of these recombinant strains were analysed after PCR amplification and their identity as *C. necator* was verified by BLAST analysis. In order to verify if the cartridge was inserted in the chromosome, PCR amplification of the *Km^r* gene was performed from the total DNA extracted from the colonies. The occurrence of a clear band (not present in the wild type strain DSM 545) revealed the presence of the gene in the chromosome of some mutants (data not shown). One of these recombinant strains, named *C. necator* sp-1, was selected for further studies.

Comparison of growth and PHA production of wild-type *C. necator* DSM 545 and recombinant *C. necator* sp-1 strain

In order to verify the effect of the above genetic modification on the reduction of PHA depolymerisation, the profiles of polymer accumulation were

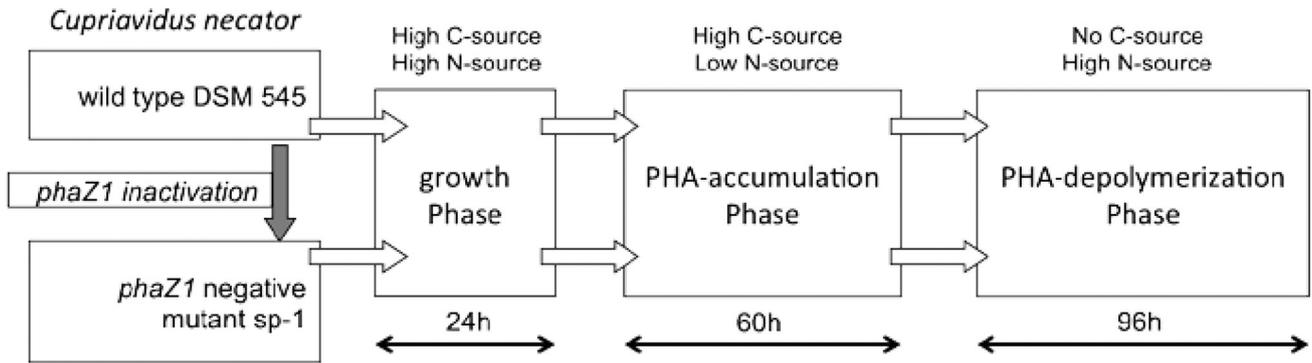


Fig. 1 – Schematic representation of the procedure adopted to evaluate PHA depolymerisation activity by wild type *C. necator* DSM545 and its derivative *phaZ1* mutant *C. necator* sp-1

analysed in *C. necator* sp-1 and its parental strain. As stated above, glycerol was adopted as the only carbon source. The DSMZ81 medium was supplemented with high carbon and low nitrogen source, conditions known to support poly(3-hydroxybutyrate) accumulation. Although sub-optimal conditions obtainable by flasks cultivation did not allow the attainment of high amounts of the polymer, both wild type and recombinant strain produced PHB, and the values were not significantly different.

Profile of poly(3-hydroxybutyrate) degradation in wild-type *C. necator* DSM 545 and recombinant *C. necator* sp-1 strain

With the aim of verifying possible advantages deriving from the inactivation of *phaZ1*, both modified and wild-type *C. necator* strains were first incubated in a carbon-rich media with low nitrogen source to promote the accumulation of the poly(3-hydroxybutyrate). After their growth and polymer accumulation, the cultures were transferred to DSMZ81 medium without any carbon source, to stimulate the strains to metabolize their internal energy/carbon reserve. Polymer content was then monitored during a period of 96 hours (Fig. 1) and the profiles of poly(3-hydroxybutyrate) degradation by wild type and recombinant *C. necator* strains are shown in Fig. 2. The reduced PHB degrading capacity of recombinant strain sp-1 suggests that the inactivation of the intracellular depolymerase gene *phaZ1*, still active in the parental strain DSM 545, may effectively result in a positive effect by preserving the polymer previously accumulated by the cell.

Conclusions

The overall objective of the whole project was to design a microbially mediated industrial process for the production of biodegradable poly(hydroxyalkanoate), thus supplying an environmentally

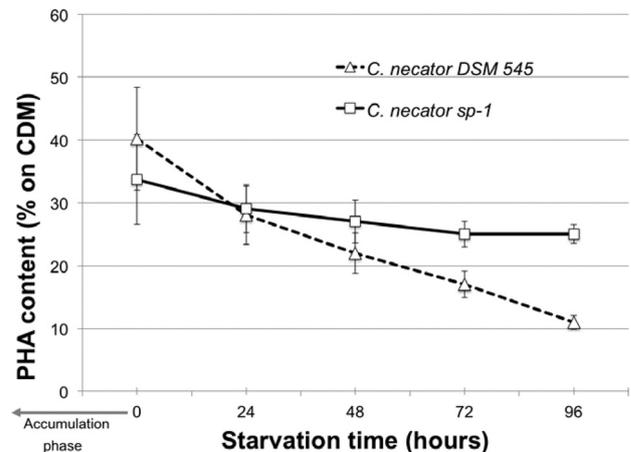


Fig. 2 – PHA depolymerisation activity by wild type *C. necator* DSM545 and its derivative *phaZ1* mutant *C. necator* sp-1 incubated under carbon starvation. Before the starvation phase, the cultures were maintained for 48–72 h with glycerol as carbon source for PHA accumulation. For culturing the recombinant strain sp-1, 100 $\mu\text{g mL}^{-1}$ kanamycin was added. The values are the mean of at least three replicates and the bars represent standard deviation.

friendly alternative to the disposal of waste from slaughterhouses, rendering industry, and waste fractions of the biodiesel production. With this aim, selected microbial strains, suitable for the production of structurally diversified PHAs under physiological stress conditions, should be available. *C. necator* represents a suitable candidate because it is able to produce high amounts of polymers and efficiently express lipase genes.

A recombinant *C. necator* strain harbouring one of the intracellular PHA-depolymerases, inactivated by chromosomal integration of a cassette containing a kanamycin resistance gene, was here obtained. Since previous studies indicated that production and mobilization of PHB occur simultaneously^{29,30} and that *phaA*, *phaB*, *phaC*, and *phaZ1a* were transcribed throughout cell growth, PHB production, and PHB degradation³², we expected to find a measurable increase in cell polymer content in the *PhaZ1* deficient strain, even from the begin-

ning of the accumulation phase. Indeed, it has been demonstrated³¹ that a *phaZ1* mutant of *R. eutropha* secretes significantly less amounts of monomeric 3HB into the culture medium, and shows significantly more and bigger PHB granules than the wild type strain during the first 12–18 hours.

However, in the present study, the two cultures (*C. necator* DSM545 and its *phaZ1* mutant strain sp-1) did not differ significantly from each other in terms of growth and polymer content. This may be partially due to the sub-optimal conditions obtainable by flasks cultivation, and especially to the long incubation time needed for allowing the cultures to reach the maximum (2–3 days).

The effect of the genetic modification on the reduction of PHA depolymerisation was then tested in the bacterial cultures previously allowed to accumulate the polymer. Under carbon starvation, while the wild type *C. necator* DSM 545 reduced the intracellular PHA to 30 % of the initial content, the transconjugant strain *C. necator* sp-1 (*phaZ1*-Km^r) maintained its PHA content at 85 % even after 96 hours incubation. This may represent an encouraging strategy to be adopted on bacterial strains selected for industrial purposes for improving the production of PHAs by preserving the bio-polymer from degradation at the end of the accumulation phase, and allowing downstream process to be performed in a wider time interval.

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List of Abbreviations and Symbols

PHA – poly(hydroxyalkanoate)
 PHB – poly(3-hydroxybutyrate)
 P(3HB-co-3HV) – copolymer Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
 LB – Luria-Bertani medium
 NB – Nutrient Broth medium
 CDM– Cell dry mass
 Km – kanamycin
 Km^r – kanamycin resistance gene
 Cm – chloramphenicol
 DSM– Deutsche Sammlung Mikroorganismen
 GC – gas chromatograph
¹HNMR – nuclear magnetic resonance

PhaZ – depolymerase enzyme
 SP-L – 5'-CATCAAGCTGCTCAAGGATG-3' primer
 SP-R – 5'-AAGAGATCTACCCGCAGCTG-3' primer
 PCR – Polymerase Chain Reaction
 BLAST – Basic Local Alignment Search Tool
 PhaZ1 – depolymerase1 enzyme
phaZ1 – depolymerase1 gene

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