



Canadian Journal of Microbiology

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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2019-0342.R2
Manuscript Type:	Note
Date Submitted by the Author:	11-Dec-2019
Complete List of Authors:	Martinez, Sarah; Institut national de la recherche scientifique, Centre Armand-Frappier Santé Biotechnologie Déziel, Eric; Institut national de la recherche scientifique, Centre Armand-Frappier Santé Biotechnologie
Keyword:	PHA granules, PHA synthase, Spectrophotometry
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

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**Changes in polyhydroxyalkanoate granule accumulation make optical density measurement
an unreliable method for estimating bacterial growth in *Burkholderia thailandensis***

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Abstract

Optical density (OD) measurement is the standard method used in microbiology for estimating bacterial concentrations in cultures. However, most studies do not compare these measurements with viable cell counts and assume that they reflect the real cell concentration. *Burkholderia thailandensis* was recently identified as a polyhydroxyalkanoate (PHA) producer. PHA biosynthesis seems to be coded by an orthologue of the *Cupriavidus necator phaC* gene. When growing cultures of wildtype strain E264 and an isogenic *phaC*- mutant, we noted a difference in their OD₆₀₀ values, although viable cell counts indicated similar growth. Investigating the cellular morphologies of both strains, we found that under our conditions the wildtype strain was full of PHA granules, deforming the cells, while the mutant contained no granules. These factors apparently affected the light scattering, making the OD₆₀₀ values no longer representative of cell density. We show a direct correlation between OD₆₀₀ values and the accumulation of PHA. We conclude that OD measurement is unreliable for growth evaluation of *B. thailandensis* because of PHA production. This study also suggests that *B. thailandensis* could represent an excellent candidate for PHA bioproduction. Correlation between OD measurements and viable cell counts should be verified in any study performed with *B. thailandensis*.

Keywords: PHA granules, PHA synthase, spectrophotometry

The soil saprophyte *Burkholderia thailandensis* was recently identified as a producer of polyhydroxyalkanoates (PHAs) (Funston et al. 2017) and then studied for the co-production of PHAs and rhamnolipid in used cook oil (Kourmentza et al. 2018). PHAs are polymers of hydroxylated fatty acids that are synthesized by different prokaryotic microorganisms as intracellular carbon storage material (Anderson and Dawes 1990). PHA granules are produced when carbon is present in excess combined with a nitrogen or phosphate limitation (Poblete-Castro et al. 2012).

PHA synthases and the genetic organisation of PHA biosynthesis gene clusters are well characterized (Rehm and Steinbüchel 1999), with two classes of PHAs principally documented: class 1 for short chain length-PHAs (scl-PHAs), products of *phaC-phaA-phaB* gene clusters featured in the *Ralstonia/Cupriavidus* genus and class 2 for medium chain length PHAs (mcl-PHAs), products of *phaC1-phaZ-phaC2* gene clusters typical of the *Pseudomonas* genus.

PHA synthases are encoded by the *phaC* homologues (Solaiman and Ashby 2005). The *phaC* gene of *B. thailandensis* was recently identified; investigating the genome of the prototypical *B. thailandensis* strain E264, a recent study (Funston et al. 2017) reported that the BTH_I2255 gene shows similarity with *Pseudomonas*-type poly-(3-hydroxyalkanoate) polymerases coded by *phaC1* and *phaC2*, with a percentage of identity of 40 and 39%, respectively. However, PhaC1 and PhaC2 are responsible for the biosynthesis of mcl-PHA, which is not consistent with the nature of PHA identified in *B. thailandensis*, that is polyhydroxybutyrate (PHB) (Kourmentza et al. 2018), the best known scl-PHA. We thus searched for a homology with a gene encoding for a scl-PHA synthase PHA. We rather found a 75% identity between BTH_I2255 and the orthologue of the *C. necator* PHA biosynthesis gene *phaC* (NC_015726.1). Furthermore, the genomic context of this gene reveals the immediate proximity of *phaA* and *phaB* orthologues, coding an acetyl-CoA acetyltransferase and an acetoacetyl-CoA

reductase, responsible for the precursors biosynthesis, with 82% and 83% identity respectively with the corresponding *C. necator* genes (CAJ92573.1 and CAJ92574.1). This suggests that a complete class 1 PHA biosynthesis machinery is present in *B. thailandensis* for the production of scl-PHAs. We indeed also found an orthologue of the *Pseudomonas aeruginosa* mcl-PHA biosynthesis gene, *phaC1* (NC_002516.2). However, it corresponds to locus tag BTH_I10418, with 74% identity. No *P. aeruginosa phaZ* gene orthologue was found, indicating that a mcl-PHAs biosynthesis pathway is not complete in *B. thailandensis*.

Optical density (OD) is a measure of the light scattered by the bacteria present in the suspension. OD measurements at 600 nm are the standard approach used in microbiology labs for estimating the bacterial concentration in a liquid culture (Koch 1961, 1970; Stevenson et al. 2016). With homogenous cultures, it is generally found that OD remains proportional to bacterial density throughout the positive phases of growth of the cultures (Monod 1942). When this requirement is fulfilled, OD determinations can provide an adequate and extremely convenient method of estimating bacterial density. However, these growth estimations can be misleading. There are relatively few studies comparing these estimations with viable cell counts (Irorere U et al. 2019). In a non-scattering sample, the attenuation in light transmission between the light source and the detector is caused by the absorbance of light by the sample. But, in a scattering sample, such as a bacterial suspension, the light reaching the detector is further reduced by the scattering of light. This decrease creates the illusion of an increase in sample absorbance, while it is actually a measure of turbidity (Matlock et al. 2011). A high concentration of intracellular PHA was known to influence the optical density measurements in bacteria, for instance in *P. putida* (Escapa et al. 2012).

We demonstrate here that PHAs accumulated as intracellular granules in *B. thailandensis* cells apparently affect the size and/or shape of the bacteria sufficiently to increase

the possibility of multiple light scattering events, making OD₆₀₀ values unreliable for growth evaluation. We also identified the *phaC* gene as responsible for the PHA production in *B. thailandensis*.

Optical density at 600 nm is not representative for growth measurement in *B. thailandensis*.

The wildtype (WT) strain was *B. thailandensis* E264. Isogenic *phaC*- (BT10416; E264 phbC148::ISlacZ-PrhaBo-Tp/FRT) and *phaC1*- (BT05079; E264 BTH_I10418-187::ISlacZ-PrhaBo-Tp/FRT) mutants were obtained from the transposon mutant library (Gallagher et al. 2013). WT, along with *phaC*- and *phaC1*- mutants were grown in 3 mL Nutrient Broth (NB; BD Difco, Mississauga, ON, Canada) medium with 2% (w/v) glycerol. The cultures were incubated at 30°C with rotation at 240 rpm in a TC-7 roller drum (New Brunswick, Canada) for 16h. These seed cultures were then diluted in flasks containing 25 mL of NB medium supplemented with 2% (w/v) glycerol to an OD₆₀₀ = 0.1 and incubated at 30°C with agitation (200 rpm) in a gyratory shaker (Infors) for 4 days. Samples were collected daily and bacterial growth was estimated by OD₆₀₀ measurements (Fig. 1A) and by viable counts (Fig. 1B), determined by plating serial dilutions on LB agar plates, and incubated for 24 hours at 37°C. Colonies were counted to determine bacterial cell count (CFU/mL) for each time points.

Interestingly, OD₆₀₀ measurements indicated that the *phaC* mutant reached lower values than the WT strain (Fig. 1A). On the other hand, loss of *phaC1* did not affect OD₆₀₀ values. This was suggesting that the *phaC* mutant has a growth defect. Unexpectedly, CFU counts revealed the concentration of live bacteria present was similar for all three strains (Fig. 1b). Thus, there is a lack of correlation between OD measures and cell density.

PHA production is responsible for the OD difference between the wildtype strain and *phaC*-mutant.

During cultivation, PHA production was verified for the three strains. Two strategies were employed. First, PHA biosynthesis was evaluated by Nile blue staining and fluorescence quantification. Indeed, Nile blue is a fluorescent dye specific for PHA granules (Ostle and Holt 1982; Page and Tenove 1996). Nile Blue staining relies on a linear correlation obtained between intracellular PHA concentrations and the corresponding fluorescence intensities (Zuriani et al. 2013).

PHA accumulation was measured as described by (Oshiki et al. 2011), with some adjustments. Culture samples of 100 μ L were collected at regular time intervals and were centrifuged for 3 min at 10,000 $\times g$. Supernatants were discarded and pellets were suspended in 100 μ L water. Samples were heated at 100°C for 10 min and transferred on ice for 5 min to lyse the cells. Samples were then centrifuged for 3 min at 10,800 $\times g$, supernatants discarded and pellets suspended in 100 μ L water. Samples were transferred to a 96-well plate and an equal volume of a 0.02% (w/v) Nile Blue (Sigma-Aldrich) solution was added to each well. After a 4 min incubation, the fluorescence intensity (excitation 490 nm / emission 590 nm) was measured using a Cytation (Biotek) multimode microplate reader.

Data show that the *phaC* mutant does not produce PHAs as no fluorescence was detected (Fig. 2A). In contrast the WT samples showed increasing fluorescence values, indicating the presence of PHA granules, as did the *phaC1* mutant, strongly suggesting that this gene is not involved in PHA production, at least under our conditions.

Next, to confirm the data obtained with the Nile blue staining, samples were prepared for observation using transmission electron microscopy (TEM). After 4 days of incubation, 1 mL of each culture were collected and transferred into a sterile 1.5 mL microcentrifuge tube. After

centrifugation (3 min, 8,000 x *g*), the supernatant was discarded and cells were fixed with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer with 3% sucrose, pH 7.4 and kept at 4°C overnight. Cells were washed three times at room temperature with 200 µL of 0.05 M cacodylate sodium with 3% sucrose pH 7.4. Cells were postfixed with 1.3% osmium tetroxide in collidine buffer. Cells were embedded in agar, which was then cut in small blocks. Post-fixation was performed with 1.3% osmium tetroxide in collidine buffer. Postfixed material was progressively dehydrated with increasing concentrations of acetone (25-100%), 30 min each and 2 x 100% acetone. The material was kept overnight in a 1:1 volume mixture of Spurr resin and acetone, then immersed twice each 2 hours, in a bath of Spurr mixtures. The block containing fixed cells was cut into small pieces, placed in BEEM capsules, filled with Spurr resin and held at room temperature for 16 hours and then polymerized at 60°C for 20-30 hours. The ultrathin sections (70-100 nm thick) were stained with supersaturated uranyl acetate in 50 % ethanol for 15 minutes, followed by lead citrate for 5 minutes. The sections were examined by a Hitachi H-7100 electron microscope with an accelerating voltage of 75 kV with AMT XR111 camera.

Under our culture conditions, the WT strain contained an average of seven big PHA granules/cell, inducing a cell deformation (Fig. 2B). WT cells were bulkier than *phaC*- mutant cells, for which TEM confirmed the absence of PHA granules (Fig. 2C).

Finally, we complemented the *phaC*- mutant by introducing the pME6000 plasmid carrying the *phaC* gene sequence (pSM2). First, *phaC* was amplified by PCR using primers: *phaC*_KpnI_fwd (CGGGGTACCGTGGGTGGTCGACTTCATG) and *phaC*_xbaI_rev (CGCTCTAGACAATCGCGTTGCATCACATAC). The insert was ligated after digestion of pME6000 and PCR product by KpnI and XbaI enzymes. The resultant plasmid from pME6000 vector and *phaC* PCR product (insert) is pSM2. The *phaC*- mutant was transformed with either the expression plasmid pSM2 or pME6000 by electroporation. PHAs biosynthesis was evaluated

after 5 days of cultivation in tubes containing 5mL of NB medium with 2% glycerol, as above. This further confirmed that the *phaC* gene is responsible for PHA biosynthesis in E264 since the presence of the complementation plasmid restored the PHA production in the *phaC*- mutant (Fig 3).

The shape or size of cells can have an impact on OD measurements (Koch and Ehrenfeld 1968). More recently, the effects of cell size variation was observed with bead suspensions and then confirmed in cultures of *E. coli* and yeast (Stevenson et al. 2016). A change in cell size throughout the growth curve of an *E. coli* grown under sub-lethal concentrations of ampicillin was demonstrated. This treatment induced a filamentation of the bacteria and thus caused a substantial deviation between OD₆₀₀ and bacterial concentration. During the initial part of the log phase, OD₆₀₀ and bacterial concentration showed the same time dependency but later the cell number remains roughly constant while the OD₆₀₀ increases (Stevenson et al. 2016). We observed a similar trend here, as when growth seemed to have stopped for both the WT and the *phaC* mutant when viable cell counts are considered, the OD₆₀₀ keeps increasing. Whether this is due to PHA-induced deformation of the cells and/or the PHA content itself is unknown. Still, data are sufficient to conclude that OD₆₀₀ is not reliable for growth assessments in culture conditions amenable to PHA biosynthesis in *B. thailandensis*.

In order to further verify this hypothesis, we evaluated PHA production in two *B. thailandensis* strains under permissive and non-permissive conditions, meaning NB medium supplemented or not with 2% glycerol. PHA are produced when excess carbon is available. Indeed, under non-permissive conditions, PHA are not produced by the *B. thailandensis* strains while the addition of glycerol allowed the biosynthesis of the biopolymers (Fig. 4E and 4F). Furthermore, the OD₆₀₀ kept increasing for both strains (Fig. 4A and 4B) when cultured in NB + glycerol while the CFU counts indicate that growth had stopped after two days (Fig. 4C and 4D).

Interestingly, CFU counts revealed that accumulation of PHA granules contributes to cellular maintenance since only under non-permissive conditions, CFU counts dropped (Fig 4C and 4D).

At least under the tested culture conditions, OD₆₀₀ was actually an indicator of PHA biosynthesis for *B. thailandensis*. Indeed, when PHA production is compared to the OD₆₀₀, the trend line is linear, indicating that PHA biosynthesis is closely correlated to the OD₆₀₀ values (Fig. 5).

In conclusion, we demonstrated that while *B. thailandensis* appears to harbour two distinct PHA biosyntheses, only class 1 *phaC* is involved in PHA production and not the class 2 *phaC1* homolog. This is consistent with the PHA composition described in a recent study (Kourmentza et al. 2018), where scl-PHA, which the authors referred to as polyhydroxybutyrate (PHB), were characterized.

Interestingly, we found here that OD₆₀₀ measurement is not reliable for growth evaluation of *B. thailandensis* when the growth conditions allow PHA production. This is consistent with other studies mentioning that turbidimetry is disturbed by the PHA amounts produced. For instance, in *Pseudomonas* species, among which are found well-known PHA producers such as *P. aeruginosa* or *P. putida*, growth measurements in term of viable cells was suggested by using gravimetric methods (Escapa et al. 2013) since dry cell mass values for growth evaluation have to be adjusted to the PHA content of cells (Escapa et al. 2012).

Also, we clearly show that *B. thailandensis* has an excellent potential as a PHA producer, probably comparable to *C. necator* (Mravec et al. 2016). PHA production in *B. sacchari* and *B. cepacia* were recently reported, with PHB contents around 88% and 74%, respectively, in a medium containing detoxified wood hydrolyzates (Kucera et al. 2017). Furthermore, Kourmentza et al (2018) reported that PHB content represents 60% of the dry cell mass when *B. thailandensis* was grown on used cooking oil (Kourmentza et al. 2018). All together, these

observations suggest that studies using OD measurements for growth assessment might be inconsistent for *Burkholderia* species.

Acknowledgments: Marie-Christine Groleau for insightful comments and Arnaldo Nakamura for TEM images. This work was supported by Discovery grant award number RGPIN-2015-03931 from the Natural Sciences and Engineering Research Council of Canada (NSERC). ED holds the Canada Research Chair in Sociomicrobiology.

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Figure 1: Optical densities at 600 nm and CFUs may not be correlated in *B. thailandensis*

cultures. E264 and *phaC*- and *phaC1*- mutants were cultured in flasks containing 25 mL of NB medium with 2% (w/v) glycerol at 30°C with agitation (200 rpm) in a gyratory shaker (Infors) for 4 days. Samples were collected daily and bacterial growth was estimated (A) by OD₆₀₀ measurements and (B) by viable counts. The error bars represent standard deviation from the mean ($n = 3$ independent cultures).

Figure 2: *phaC* mutant of *B. thailandensis* E264 do not produce PHAs. (A) PHA production for the wild type strain and the *phaC*- and *phaC1*- mutants measured by Nile blue staining during 4 days. (B) and (C) are TEM images (x10.000) for the wildtype strain and the *phaC*- mutant respectively.

Figure 3: Complementation of the *phaC*- mutant restored PHA production.

phaC- mutant complemented with the pSM2 plasmid carrying the *phaC* gene sequence was cultured in tubes containing 5mL of NB medium with 2% (w/v) glycerol at 30°C with agitation (200 rpm) in a rotary shaker. Wild type and *phaC*- mutant strains with pME6000 were cultured too in the same conditions as controls. PHA production was measured by Nile blue staining after 5 days. Data analysed using a one-way ANOVA with post hoc. Dunnett's multiple comparisons tests (**** $p < 0.0001$)

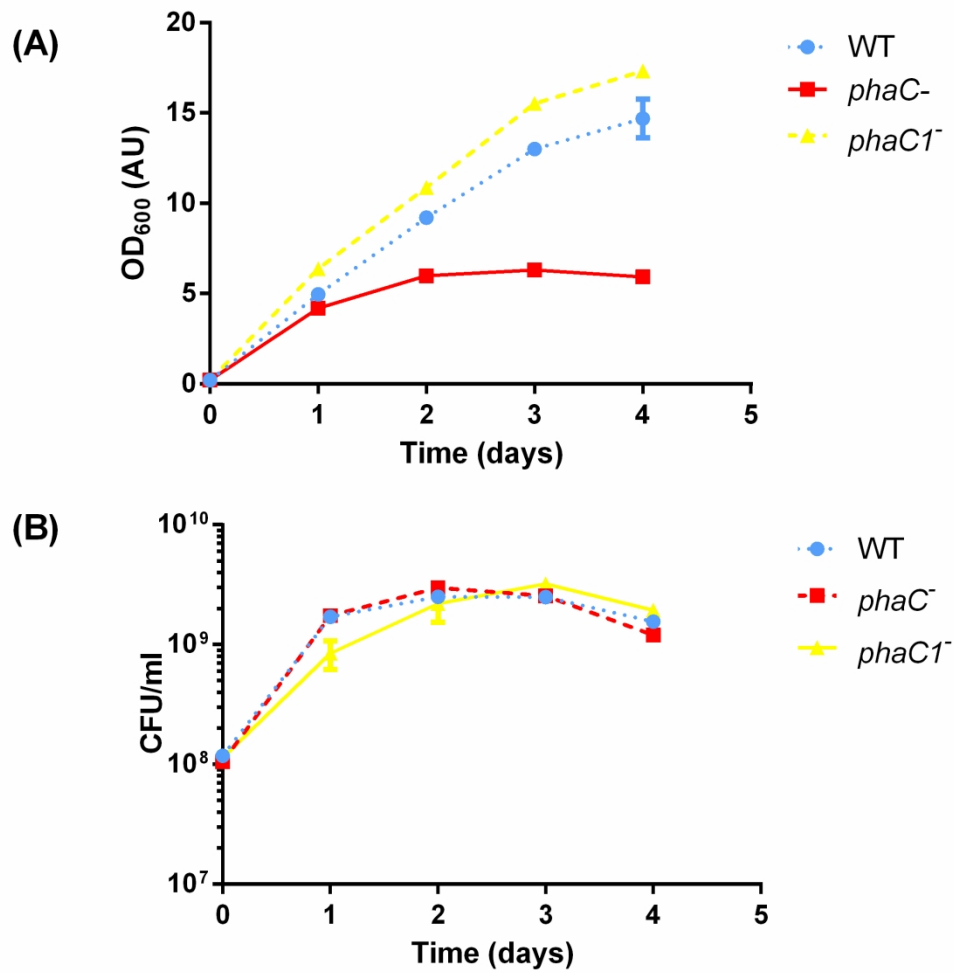
Figure 4: Optical density can be used for growth evaluation under non permissive conditions for PHA biosynthesis.

E264 and ED862 were cultured in flasks containing 25 ml of NB medium with or without addition of 2% (w/v) glycerol at 30°C with agitation (200 rpm) in a gyratory shaker (Infors) for 4 days. Samples were collected daily and bacterial growth was estimated for both strains (A) and (B) by OD₆₀₀ measurements

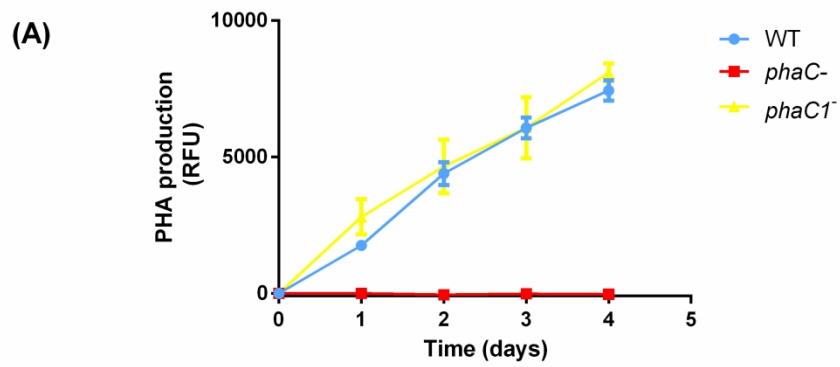
and (C) and (D) by viable counts. Finally the PHA production was evaluated by Nile blue staining (E) and (F). The error bars represent standard deviation from the mean ($n = 3$ independent cultures).

Figure 5: PHA biosynthesis is correlated to OD_{600} measures. PHA biosynthesis is represented depending on the OD_{600} values measured during the growth of the E264 strain in NB medium supplemented with 2% glycerol. The error bars represent standard deviation from the mean ($n = 3$ independent cultures).

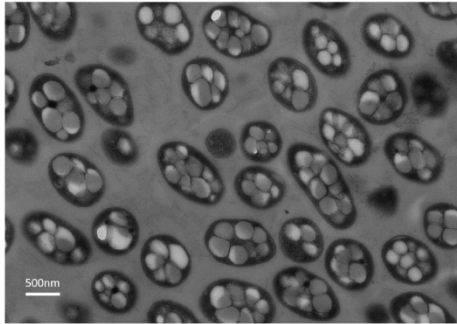
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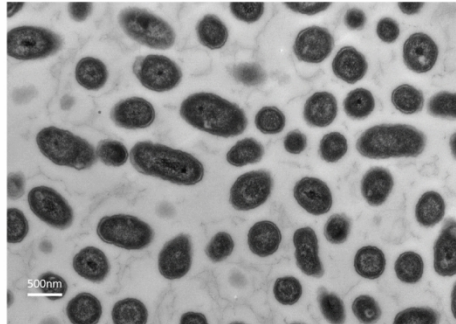
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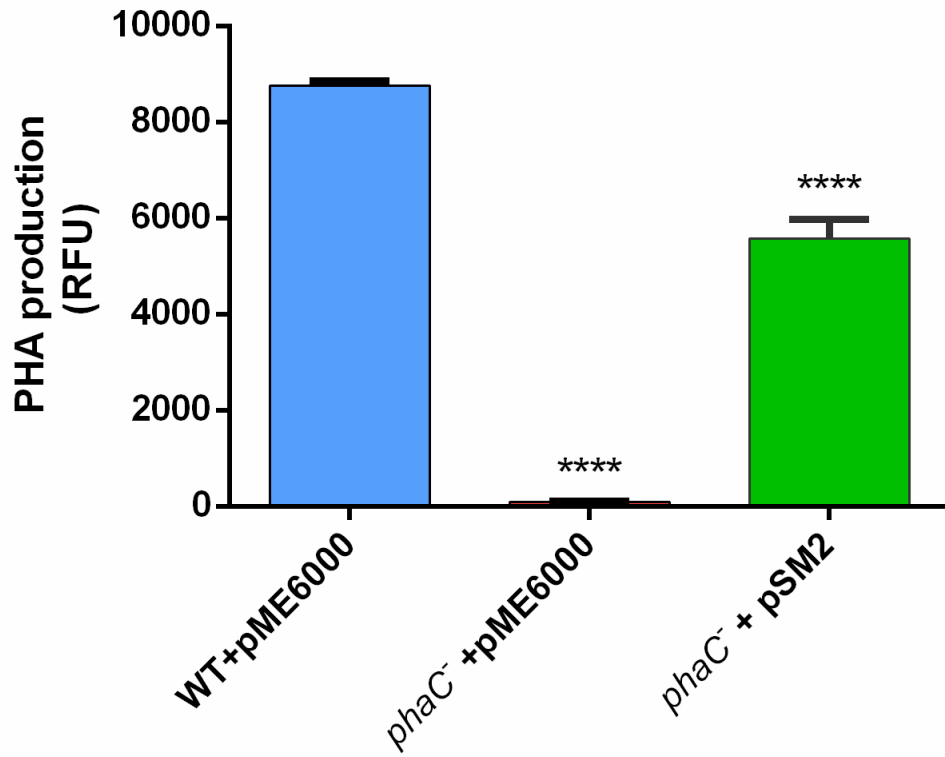
(B)



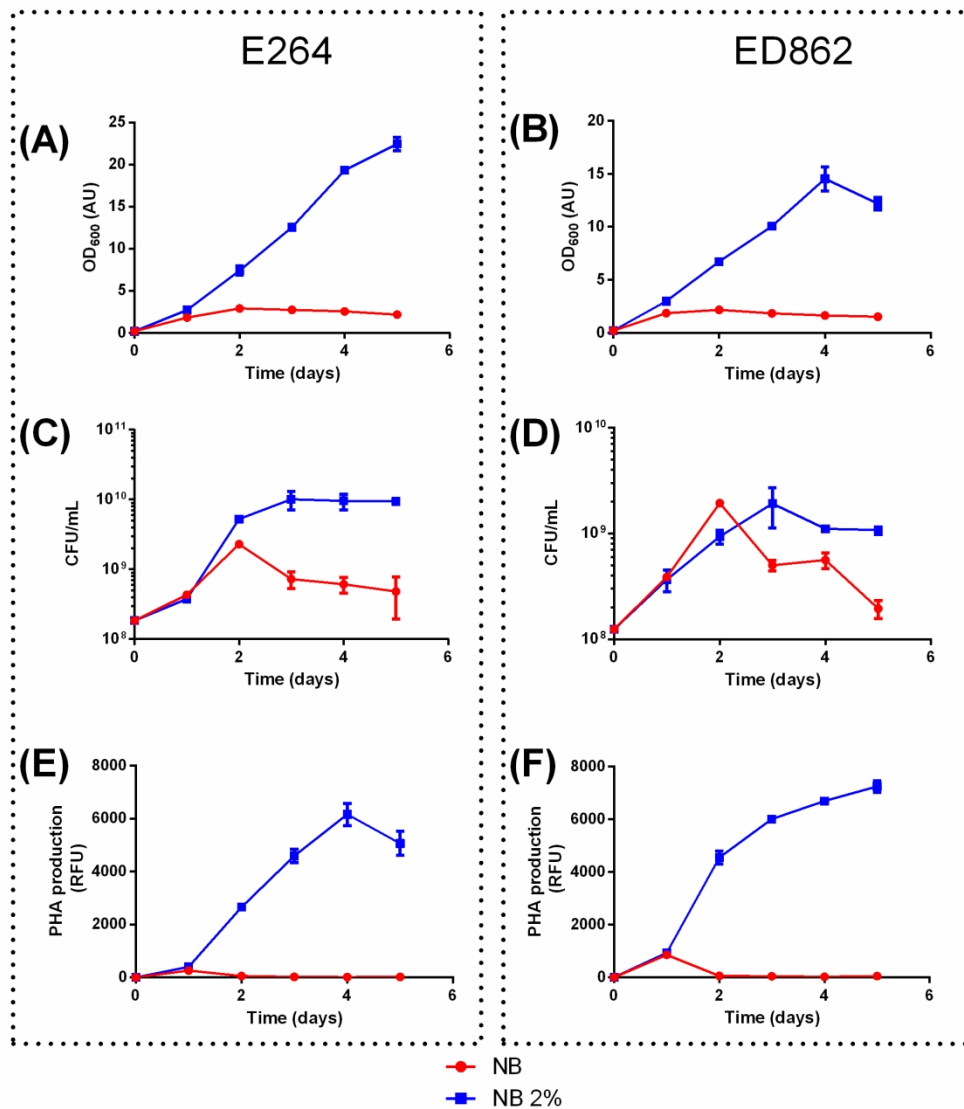
(C)



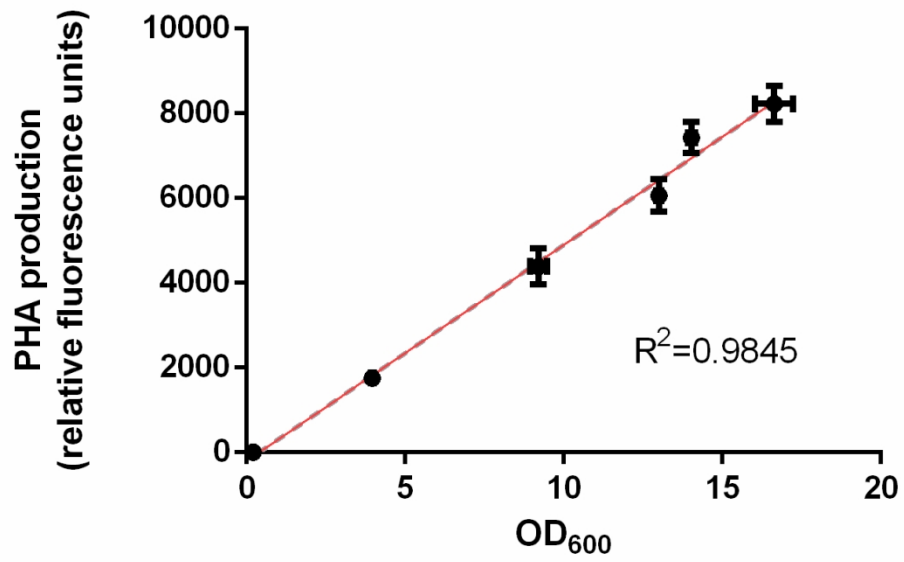
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