The effect of polyphosphate kinase (*ppk*) deletion on polyhydroxyalkanoate accumulation and carbon metabolism in *Pseudomonas putida* KT2440

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27 ***Summary***

28 The primary enzyme involved in polyphosphate (polyP) synthesis, polyphosphate kinase (*ppk*),
29 has been deleted in *Pseudomonas putida* KT2440. This has resulted in a 3 to 6 fold reduction in
30 polyhydroxyalkanoate (PHA) accumulation compared to the wild-type under conditions of nitrogen
31 limitation, with either temperature or oxidative (H_2O_2) stress, when grown on glucose. The accumulation
32 of PHA by *Δppk* mutant was the same as the wild-type under nitrogen limiting growth conditions. There
33 was no difference in polyP levels between wild-type and *Δppk* strains under all growth conditions tested.
34 In the *Δppk* mutant proteome, polyphosphate kinase (PPK) was undetectable but up-regulation of the
35 polyP associated proteins polyphosphate ATP/NAD kinase (PpnK), a putative polyphosphate AMP
36 phosphotransferase (PP_1752), and exopolyphosphatase (PPX) was observed. *Δppk* strain exhibited
37 significantly retarded growth with glycerol as carbon and energy source (42 h lag period compared to 24
38 h in wild-type strain) but similar growth to the wild-type strain with glucose. Analysis of gene
39 transcription revealed down-regulation of glycerol kinase (*glpK*) and the glycerol facilitator (*glpF*)
40 respectively. Glycerol kinase protein expression was also down-regulated in the *Δppk* mutant. The
41 deletion of *ppk* did not affect motility but reduced biofilm formation. Thus, the knockout of the *ppk* gene
42 has resulted in a number of phenotypic changes to the mutant without affecting polyP accumulation.

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50 ***Introduction***

51 Polyphosphate (polyP) is an inorganic polymer composed of chains of tens or hundreds of
52 phosphate (P_i) residues linked via high energy phosphoanhydride bonds (Kulaev, 1975). PolyP is
53 produced through the action of the polyphosphate kinase enzyme (PPK) which is encoded by the *ppk* gene
54 (Achbergerová and Nahálka, 2011). PolyP has been implicated in several roles in bacteria including as a
55 substitute for ATP for the phosphorylation of various sugars, nucleosides, coenzyme precursors and
56 proteins with its levels fluctuating according to nutritional availability (Skorko, 1989; Kuroda and
57 Kornberg, 1997; Kornberg *et al*., 1999). This has led to suggestions that it is likely a regulatory molecule
58 (Kulaev *et al*., 1999). The role of polyP in stress response has been extensively reported for *Escherichia*
59 *coli* and *Pseudomonas aeruginosa* (Rao *et al*., 1998; Rashid and Kornberg, 2000; Tsutsumi *et al*., 2000;
60 Fraley *et al*., 2006; Brown and Kornberg, 2008; Schurig-Briccio *et al*., 2009,).

61 We have previously described the dual accumulation of both polyP and the biodegradable carbon-
62 based polymer medium chain length PHA (mcl-PHA), in a number of *Pseudomonas* strains including the
63 biotechnologically important strain *P. putida* KT2440 (Tobin *et al*., 2007). Both polymers are
64 accumulated concurrently for the first 24 h of growth after which time polyP is degraded but mcl-PHA
65 accumulation continues. In an attempt to determine if the polyP accumulation is critical or benefits mcl-
66 PHA accumulation, as is the case for polyhydroxybutyrate (PHB) (Smolders *et al*., 1995; Mino *et al*.,
67 1998), in this work, we have deleted the *ppk* gene in *P. putida* KT2440. Such a gene deletion has been
68 shown to dramatically reduce polyP levels in *E. coli* and *P. aeruginosa* (Fraley *et al*., 2006; Schurig-
69 Briccio *et al*., 2009). However, the *ppk* gene deletion mutant of *P. putida* KT2440 accumulated normal
70 levels of polyP. This finding provided us with a unique opportunity to study the effect of the *ppk* gene
71 deletion on the physiology of *P. putida* KT2440 cells accumulating normal levels of polyP under normal
72 growth and stressful conditions. We have previously shown that stress proteins were up-regulated during
73 mcl-PHA accumulation (Nikodinovic-Runic *et al*., 2009) and thus wished to examine the ability of the
74 *ppk* mutant to accumulate PHA under stress conditions.

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75 Recently, Escapa and co-workers suggested the phosphorylation state of a carbon substrate,
76 glycerol, affected the lag period of growth for *P. putida* KT2440 on this substrate (Escapa *et al*., 2013).
77 The metabolism of carbon substrates such as glucose and glycerol involve phosphorylation, for instance,
78 the first step in glycerol metabolism is the phosphorylation of glycerol to glycerol-3-phosphate
79 (Schweizer *et al*., 1997). In order to gain insight into the effect of *ppk* gene deletion on carbon
80 metabolism we examined the growth of the mutant on glycerol and glucose. Therefore, the current
81 manuscript describes a global physiological effect of *ppk* gene deletion with mcl-PHA accumulation,
82 stress response, and carbon metabolism affected in *P. putida* KT2440 cells accumulating normal levels of
83 polyP.
84
85 ***Results and discussion***
86 *Effect of ppk deletion on polyP accumulation*
87 It was vital to determine if the *Δppk* mutant was still capable of producing the polyP polymer in
88 the absence of a functioning PPK enzyme. As polyP is associated with stress response, levels were
89 determined under oxidative and temperature stress as well as under non-stress and limited phosphate
90 conditions. Relative polyP accumulation by wild-type and mutant strains were analysed at various
91 starting concentrations of KH_2PO_4 (0.07, 1, 10, 36 mM) over a 48 h period (Fig. 1A). Despite the
92 knockout of the *ppk* gene, the mutant was still capable of accumulating polyP. Both wild-type and mutant
93 strain accumulated 2 fold and 4 fold higher levels of polyP respectively, with 0.07 mM phosphate in the
94 medium compared to other concentrations (1, 10, 36 mM). In general polyP levels of wild-type and
95 mutant were shown to accumulate over the first 15 h of growth and reached a maximum at 24 h before a
96 gradual decline over the second 24 h (Fig. 1B). While certain growth conditions reduced polyP levels,
97 there were broadly similar for both *Δppk* mutant and wild-type after 48 h of growth under oxidative and
98 temperature stress (Fig. 2A and Fig. 2B) and non-stress (Fig. 1A and Fig. 1B) growth conditions. The
99 one exception was that the mutant strain (*Δppk*) accumulated 1.8 fold less polyP compared to the wild-

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100 type strains after 48 h of incubation at 37 °C (Fig. 2B). Deletion of the *ppk* gene in other bacteria such as
101 *P. aeruginosa* and *E. coli* has been shown to reduce polyP accumulation compared to the wild-type strain
102 and have a negative effect on bacterial survival, stress response (Rao and Kornberg, 1996), biofilm
103 formation and motility (Shi *et al*., 2004). Interestingly the knockout of the *ppk* gene in *P. putida* KT2440
104 did not affect the level or profile of polyP accumulation over time (Fig. 1B).

105 To elucidate how the mutant was capable of producing polyP without a functioning PPK, various
106 proteins associated with the metabolism of polyP were targeted for the expression analysis. No
107 production of the PPK protein was detected in the Δ*ppk* mutant at any stage of growth with glycerol (Fig.
108 3, Supporting Table 1). However, several polyP associated proteins were up-regulated in the Δ*ppk* mutant
109 during mid and late log phases of growth. These included a probable inorganic polyphosphate/ATP-NAD
110 kinase, a polyphosphate:AMP phosphotransferase and exopolyphosphatase (PPX) (Fig. 3, Supporting
111 Table 1). A subset of 30S ribosomal proteins, used as controls, were unaffected by the *ppk* knockout
112 (Supporting Table 1).

113 It is possible that these proteins are up-regulated in the absence of a functioning PPK and may
114 explain why the polyP polymer is still produced in the mutant. Itoh and Shiba (2004) reported that AMP
115 phosphotransferases can form polyP in vitro and thus the replacement of PPK activity is possible by AMP
116 phosphotransferase. The presence of elevated levels of exopolyphosphatase in the mutant is surprising as
117 polyP levels are the same as wild-type (Fig. 1AB). The up-regulation of a hydrolysing enzyme in a cell
118 accumulating polyP would promote a futile energy consuming cycle. It may be possible that the PPX can
119 catalyse a reverse reaction and produce polyP however this has not been reported in other bacteria to date
120 (Akiyama *et al*., 1993; Wurst and Kornberg, 1994). Importantly, no detectable level of PPK protein or
121 *ppk* gene transcript was observed in the proteomic and transcription analysis.

122

123 *Effect of ppk deletion on PHA accumulation*

124 PolyP has been implicated in stress response and adaptation. In order to understand the effect of
125 the *ppk* knockout we examined the ability of the *Δppk* mutant to accumulate the important biopolymer

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126 PHA under both non-stress and stress conditions (oxidative and temperature), in comparison to the wild-
127 type.

128 Under non-stress conditions, with glucose present as sole carbon source in the presence of
129 limiting nitrogen concentrations, the wild-type strain produced 0.62 g l^{-1} cell dry weight (CDW) and 35 %
130 PHA (0.25 g l^{-1}). The mutant grew to similar levels (0.65 g l^{-1}) and produced PHA to 32 % (0.21 g l^{-1}).

131 While both the wild-type and mutant showed a decrease in PHA accumulation in the presence of
132 0.5 mM H_2O_2 the mutant strain (*Δppk*) accumulated 6 fold less PHA compared to the wild-type strain
133 (Fig. 4A). Under the same growth conditions, the *Δppk* mutant strain achieved 1.4-fold lower biomass
134 compared to the wild-type (Fig. 4C). When the hydrogen peroxide (H_2O_2) concentration was increased to
135 1 mM both strains were dramatically inhibited in growth and no detectable PHA was accumulated due to
136 low biomass (Fig. 4AC).

137 PHA accumulation in the *Δppk* mutant was 2.1 and 3.0 fold lower compared to the wild-type
138 strain at 20 °C and 37 °C respectively when supplied with glucose as the sole carbon and energy source
139 (Fig. 4B). Both, wild-type and *Δppk* mutant strains, accumulated similar levels of PHA at 25 °C and 30
140 °C with glucose as carbon source. No significant difference in biomass was observed between both
141 strains at any of the temperatures tested after 48 h of incubation with glucose as the carbon and energy
142 source (Fig. 4D). Furthermore, both wild-type and mutant strains were found to have similar growth
143 characteristics when grown on glucose under nitrogen limitation (Supporting Fig. 1), implying that the
144 reduced PHA accumulation of the mutant under conditions of dual stress is not due to delayed growth.

145 We have previously reported the production of stress proteins by *P. putida* CA-3 during PHA
146 accumulation (Nikodinovic-Runic *et al.,* 2009) and the negative impact of deletion of a stress protein Clp
147 protease subunit ClpA on PHA accumulation (Goff *et al*., 2009). While inorganic nutrient limitation is
148 considered a stress, alone it is insufficient to reduce the PHA accumulating capacity of the *Δppk* mutant.
149 However, the presence of an additional stress (e.g. temperature, H_2O_2) negatively impacts PHA

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150 accumulation by the *Δppk* mutant far more than the wild-type strain (Fig. 4A and Fig. 4B) suggesting that
151 the *ppk* gene product plays a role in PHA accumulation from glucose under conditions of dual stress.
152
153 *Effect of ppk deletion on glycerol metabolism*
154 Glycerol is an important carbon source for the production of biological polymers such as PHA.
155 Its metabolism in *P. putida* is reliant on phosphorylation by the action of glycerol kinase. Accumulation
156 of glycerol-3-phosphate up-regulates glycerol metabolism by interacting with the negative regulator GlpR
157 (Schweizer and Po, 1996). This step is rate limiting and contributes to a long lag phase (over 24 h) when
158 *P. putida* was grown in the presence of glycerol (Fig. 5A). Due to the importance of this phosphorylation
159 step we examined the link between polyP and glycerol metabolism with regard to PHA accumulation.
160 When grown in the presence of 20 mM glycerol the *Δppk* mutant achieved 0.17 g l^{-1} of biomass
161 compared to 0.59 g l^{-1} for the wild-type strain after 48 h of incubation (Fig. 5A). There was a
162 significantly prolonged lag phase of 42 h for the *Δppk* mutant. In addition the Δ*ppk* mutant was unable to
163 produce any PHA polymer over this time (Fig. 5B). Transcription levels for key glycerol metabolic genes
164 were determined by quantitative PCR and compared between wild-type and *Δppk* strains during early-log
165 growth. All values are given as fold changes for the mutant compared to the wild-type levels using *rpoN*
166 sigma factor as the internal control. It was found that *glpK* (glycerol kinase) was down-regulated 2.0 fold
167 thus potentially retarding the formation of glycerol-3-phosphate. The *glpF* (glycerol uptake facilitator)
168 was also down-regulated 2.5 fold in the *Δppk* mutant compared to the wild-type which could have serious
169 implications on glycerol uptake rate and is likely to play a role in the elongated lag phase observed for the
170 *Δppk* strains. Previously a *glpF* transposon knockout in *E. coli* was seen to have a glycerol transport
171 negative phenotype (Sweet *et al*., 1990). The *glpD* (glycerol-3-phosphate dehydrogenase) gene was
172 down-regulated 1.4 fold in the mutant. However, this fold difference may be too low to be considered as
173 biologically relevant.

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174 Proteomic analysis revealed that the glycerol kinase protein was down-regulated throughout mid
175 and late log stages of growth (Fig. 3, Supporting Table 1). According to spectral count estimation the
176 glycerol dehydrogenase protein was expressed to the same levels in the wild-type and Δ*ppk* mutant at
177 early log growth and at higher levels in the *Δppk* mutant in the mid and late log phase of growth
178 (Supporting Table 1). However the highest expression levels of the dehydrogenase enzyme in the *Δppk*
179 mutant (late log) and wild-type (mid log) were the same. The production levels of both proteins correlate
180 well with the observed transcript data mentioned above during early log growth, however, the GlpF
181 protein was not detected during expression analysis. A subset of 30S ribosomal proteins were used as
182 controls and were unaffected by the *ppk* knockout (Supporting Table 1).
183

184 *Complementation of* Δ*ppk mutant*

185 In order to ensure that the phenotypic effects observed were not related to polar effects due to the
186 mutagenesis process, a complemented strain of the *Δppk* mutant was generated by expressing the *ppk*
187 gene on a broad host range expression vector pJB861in the *Δppk* mutant. *P. putida* KT2440 Δ*ppk*
188 complemented mutant grew to 86 % of the wild-type CDW (Supporting Fig. 2A) in the presence of the *m*-
189 toluic acid inducer and 20 mM glycerol and recovered just under half of the PHA accumulating ability of
190 the wild-type (Supporting Fig. 2B). The *P. putida* KT2440 Δ*ppk* mutant failed to reach higher levels of
191 biomass or PHA in the presence of the inducer compared to the control (no inducer).
192

193 *Effect of ppk deletion on biofilm development and motility*

194 PolyP is reported to have a significant impact on bacterial biofilm development (Fraley *et al*.,
195 2006) and motility (Rashid *et al*., 2000). However, *P. putida* KT2440 *Δppk* accumulates normal levels of
196 polyP at 30 °C and thus it provided an opportunity to examine the effect of *ppk* gene deletion, in the
197 presence of polyP, on biofilm formation and motility. Biofilm formation was 1.6 fold lower for the *Δppk*
198 mutant compared to wild-type when incubated for 48 h on MSM supplemented with 0.4 % glucose at 30

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199 °C (Supporting Fig. 3). However, motility was unaffected as both wild-type and *Δppk* mutant exhibited
200 similar motility to each other at all temperatures tested with motility highest for both strains at 30 °C
201 (Supporting Fig. 4).
202
203 ***Conclusion***
204 Since PHA accumulation is affected in the *Δppk* mutant it suggests that the accumulation of
205 polyP and mcl-PHA are not interdependent in *P. putida* KT2440 which differs from short chain length
206 PHA (PHB) and polyP where accumulation in cells is interdependent (Lee and Choi, 1999). In the *Δppk*
207 mutant strain a number of cellular functions are compromised (stress response, PHA accumulation, and
208 glycerol metabolism), gene transcription reduced (*glpf*, *glpk*), and protein expression altered (GlpK, PPX,
209 PpnK) suggesting that the *ppk* gene or its product in *P. putida* KT2440 has a regulatory function. The
210 same levels of polyP in the *Δppk* mutant and wild-type under stress and non-stress growth conditions
211 suggests there is either not a link between the *ppk* gene and polyp accumulation or other genes or proteins
212 can substitute for the role of polyphosphate kinase in *P. putida* KT2440.
213
214
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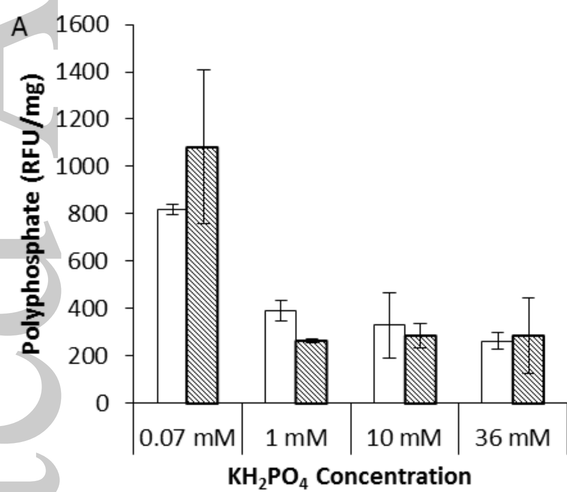

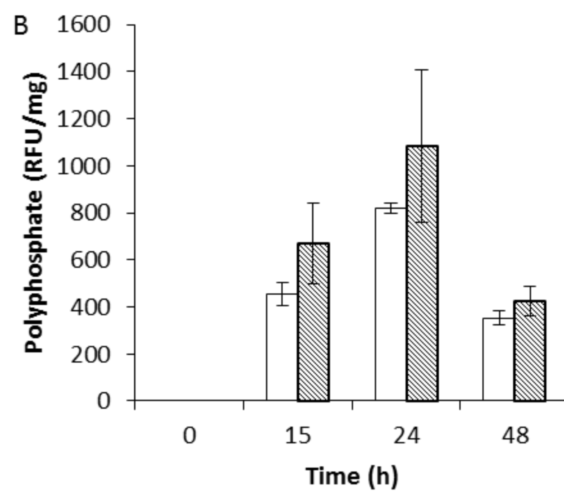

□KT2440 wild-type ▨KT2440 *Δppk*

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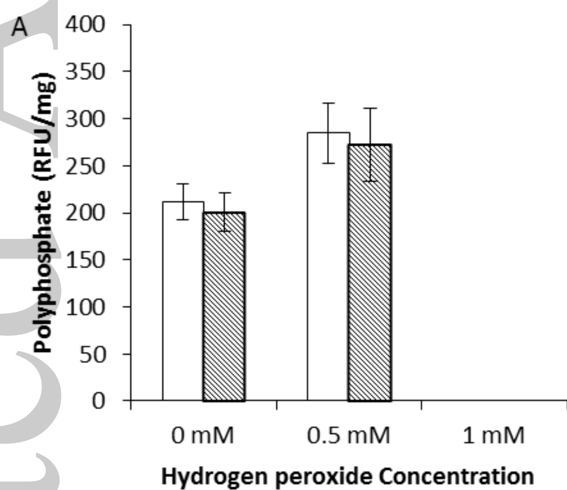
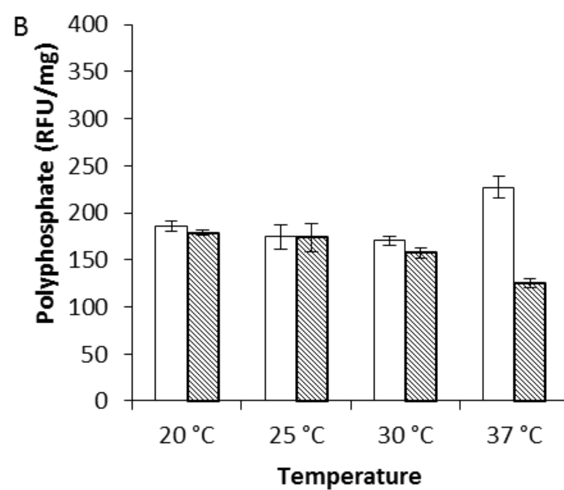
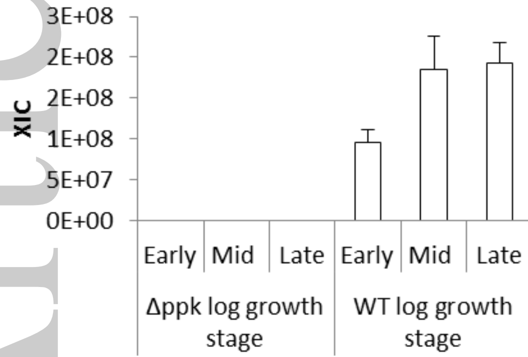


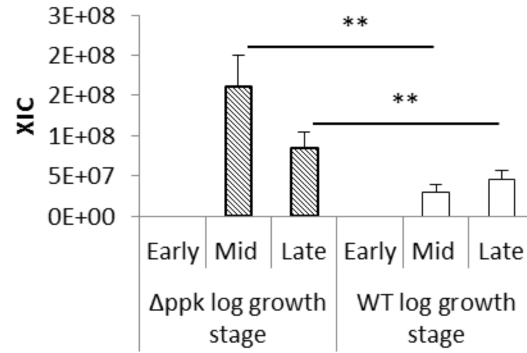


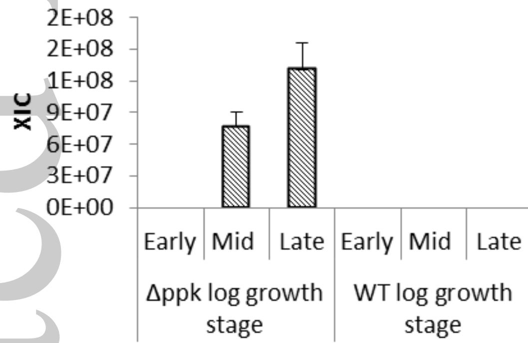
KT2440 wild-type KT2440 *Δppk*

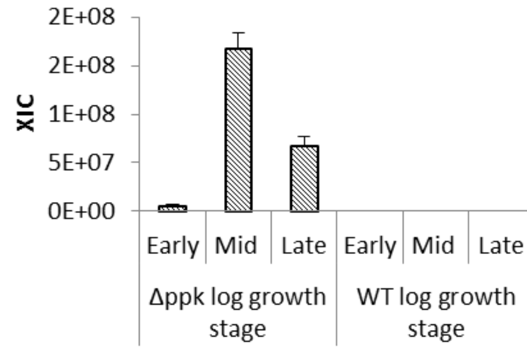
emi4_12076_f2.tif

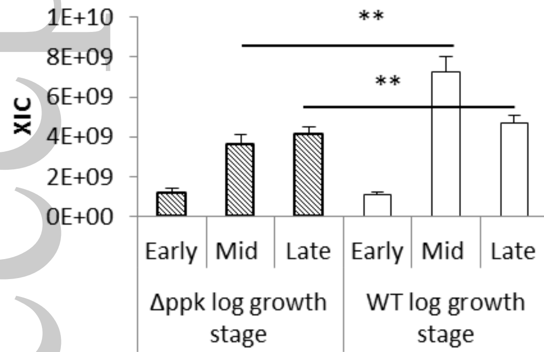
Accepted Article

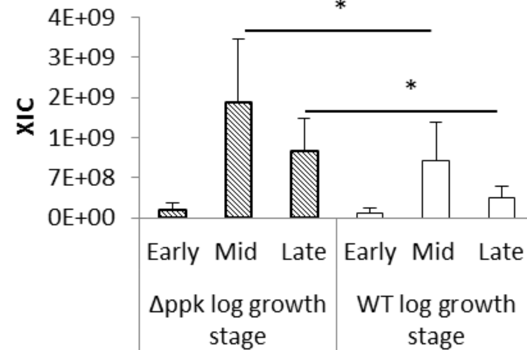








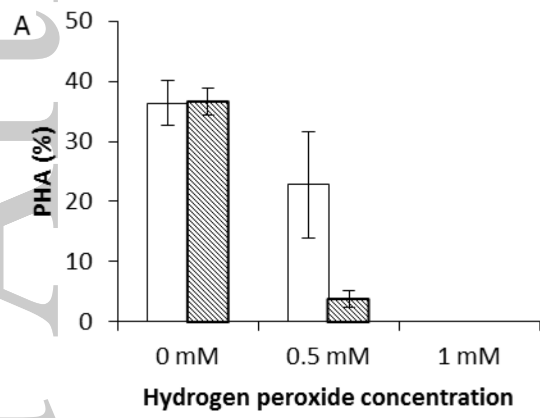


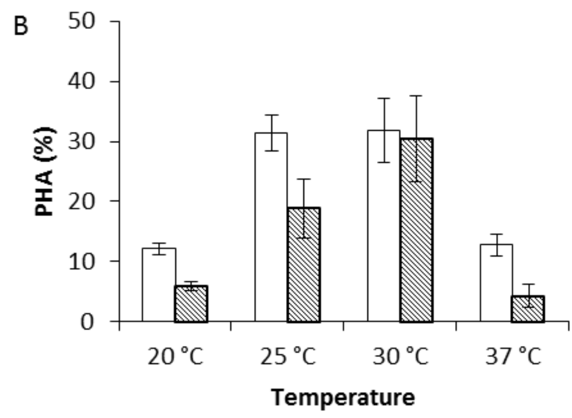


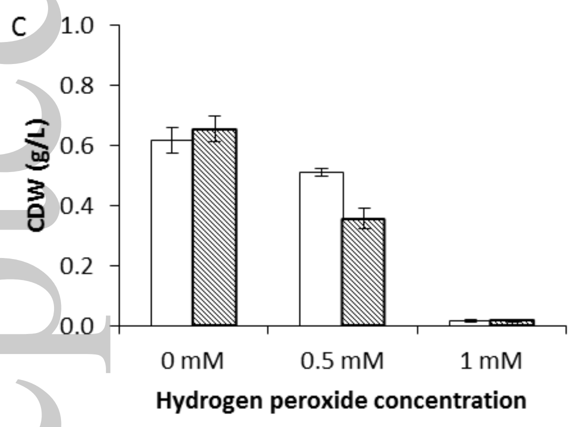
□KT2440 wild-type ▨KT2440 *Δppk*

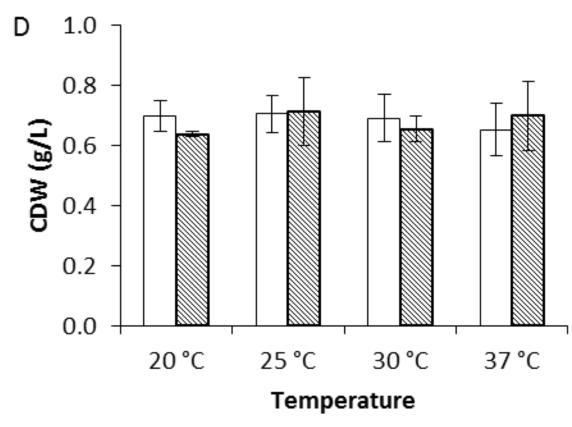
emi4_12076_f3.tif

Accepted Article





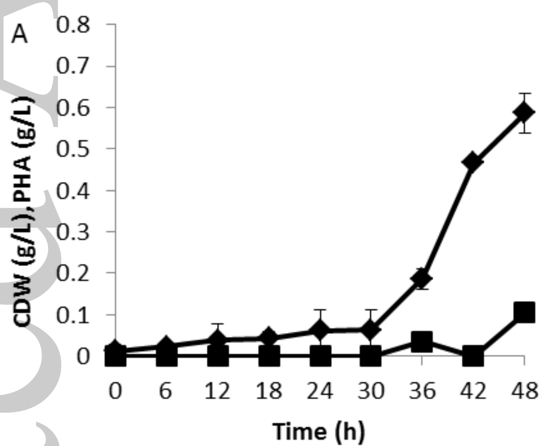


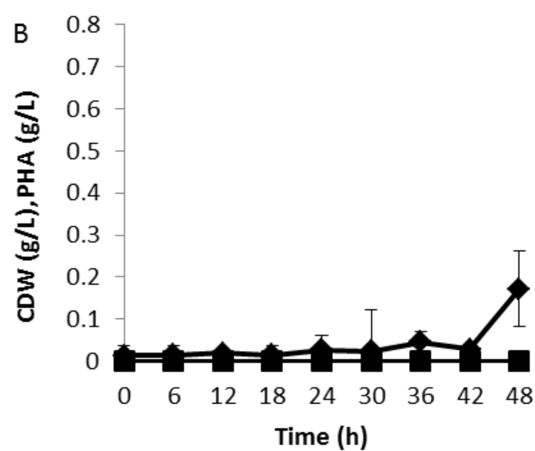


□KT2440 wild-type ▨KT2440 *Δppk*

emi4_12076_f4.tif

Accepted Article





◆ CDW (g/L) ■ PHA (g/L)

emi4_12076_f5.tif