



28 Enzyme Kinetic Model (EKM) of respiratory oxygen consumption based on  
29 the substrate control of the ETS. It postulates that the upper limit of  $R$  is set  
30 by the maximum velocity,  $V_{max}$ , of complex I of the ETS and the temperature,  
31 and that the substrate availability,  $S$ , modulates  $R$  between zero and this  
32 upper limit. Kinetics of this thermal-substrate regulation are described by  
33 the Arrhenius and Michaelis-Menten equations. The EKM equation takes  
34 the form  $R = \frac{ETS [S] e^{\frac{-E_a}{R_g T}}}{K + [S]}$  where  $R_g$  is the molar gas constant and  $K$  is the  
35 Michaelis-Menten constant.

36 Here, we apply the EKM and the MTE to predict a respiration time-  
37 profile throughout the exponential, steady state, and nutrient-limited phases  
38 of the marine bacteria *Pseudomonas nautica* and *Vibrio natriegens* in acetate-  
39 based cultures. Both models were tested by comparing their output with  
40 the measured  $R_{O_2}$  time-profile. The MTE predicted respiration accurately  
41 only in the exponential growth phase, but not during the nutrient limitation  
42 part of the stationary phase. In contrast, the EKM worked well throughout  
43 both physiological phases as long as the modelled substrates fall with the  
44 declining carbon source. Results support the theoretical bases of the EKM.  
45 We conclude that the EKM holds promise for predicting respiration at the  
46 different physiological states and time-scales important to microbiological  
47 studies.

48 *Keywords:* ETS, modeling respiration, MTE, Oxygen consumption

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## 49 **1. Introduction**

50 First principles-based models of physiological processes are rare. For res-  
51 piration we have statistically based allometric equations relating respiration

52 to body size, body surface, or biomass (Weibel, 2002; Brown et al., 2004;  
53 Allen and Gillooly, 2007). However, we have few models relating respiration  
54 to the fundamental chemical principles and processes that control it. Equa-  
55 tions for respiration based on biochemical principles and properties such as  
56 enzyme activities and substrate concentrations are not unreasonably difficult  
57 to conceive, but have rarely been formulated as they have been for nitrogen  
58 uptake and photosynthesis (Packard et al., 1971; Farquhar et al., 1980). Such  
59 models would provide a means for calculating physiological rates when di-  
60 rect measurements are impractical. Here we present a biochemical model of  
61 respiratory oxygen consumption based on the substrate control of the respi-  
62 ratory electron transport system. This model follows the equations designed  
63 to calculate phytoplankton nitrate uptake (Packard et al., 1971), and bacte-  
64 rial respiration (Packard et al., 1996a,b; Roy and Packard, 2001). They are  
65 conceptually similar to Farquhar’s photosynthetic model (Farquhar et al.,  
66 1980).

67 The derivation of these equations is based on the assumptions that (1) res-  
68 piration is the direct result of intracellular activity of the electron transport  
69 system (ETS) following a definable stoichiometry; (2) regulation of the ETS,  
70 and hence respiration ( $R_{O_2}$ ) at the physiological level, is controlled by the  
71 NADH Dehydrogenase (EC 1.6.99.3.); and NADPH transferase (EC 1.6.1.1.)  
72 at the entrance to the ETS; and (3) that the reactions of these enzymes obey  
73 the rules of enzyme kinetics.

74 The Metabolic Theory of Ecology (MTE) provides an alternative model  
75 of respiration. Since the middle 90’s articles proposing the MTE as a new  
76 unified theory for biology (Whitfield, 2005, 2006), based on the allometric

77 equation of Kleiber (1932), have appeared in key journals (Brown et al.,  
78 2000, 2004; Brown and West, 2000; Enquist et al., 1998, 2000; West et al.,  
79 1997, 2000, 2001). The proponents argue convincingly that anabolic and  
80 catabolic metabolism are determined by biomass, temperature, and the flux  
81 of elemental materials through an organism. They find parallel fractal scaling  
82 in animal and plant distribution networks and circulatory systems as well as  
83 similar thermodynamics and metabolic kinetics to explain the widespread  
84 allometry with biomass. The MTE argues that respiration in all organisms,  
85 including bacteria, can be calculated from biomass (M), temperature (T),  
86 and a stoichiometric factor (C) that controls the uptake of minerals and  
87 nutrients (Brown et al., 2004). The MTE algorithm is:

$$R = C M^{\frac{3}{4}} e^{\frac{-E_a}{kT}} \quad (1)$$

88 The biomass (M), with an exponent  $b = \frac{3}{4}$ , is the core of Kleiber's law,  
89  $R = aM^b$  (Kleiber, 1932, 1961; Whitfield, 2006), where  $a$  is a constant. In the  
90 MTE this constant,  $a$ , is folded into the MTE constant,  $C$ . In Kleiber's law  
91 when  $M^b = 1$ ,  $a = R$  and thus the units of  $a$  and  $R$  are the same. Kleiber's  
92 Law holds over a range of  $10^{20}$  (Hochachka and Somero, 2002; Whitfield,  
93 2006). The temperature dependency is based on the Boltzmann factor,  $e^{\frac{-E_a}{kT}}$ ,  
94 where  $E_a$  is the Arrhenius energy of activation (for respiration,  $E_a \approx 0.65$  eV  
95 (Allen and Gillooly, 2007)),  $k$  is the Boltzmann constant for an atom or  
96 molecule ( $0.33 \times 10^{-23}$  cal K<sup>-1</sup> or  $8.62 \times 10^{-5}$  eV K<sup>-1</sup> (Allen and Gillooly,  
97 2007)), and T is the temperature in Kelvin. For the stoichiometric factor, or  
98 nutrient availability, the MTE uses the constant,  $C$  (or  $b_0$ , (Gillooly et al.,  
99 2006)). This MTE has been applied to secondary production, respiration

100 (Gillooly et al., 2001), growth, and developmental time (Gillooly et al., 2002,  
 101 2003), etc. in both plants and animals.

102 The Enzyme Kinetic Model (EKM) argues that each metabolic process  
 103 is controlled by the maximum velocity ( $V_{max}$ ) of the enzyme reaction that  
 104 controls the process, the temperature ( $T$ ), and the substrate availability ( $S$ ).  
 105 Focused on respiration ( $R_1$ ), at temperature  $T_1$ , the EKM equation takes the  
 106 form:

$$R_1 = \frac{ETS_0 [S] e^{\frac{-E_a}{R_g(T_1-T_0)}}}{K + [S]} \quad (2)$$

107  $ETS_0$  is the potential respiration rate (in the same units as the physio-  
 108 logical rate,  $R_1$ ) but measured at another temperature,  $T_0$ .  $ETS_0$  is also the  
 109 *in vitro* activity of the respiratory electron transport system, its  $V_{max}$  (*sensu*  
 110 Michaelis-Menten). The expression  $e^{\frac{-E_a}{R_g(T_1-T_0)}}$  is from the Arrhenius Equa-  
 111 tion where  $E_a$  is the Arrhenius energy of activation ( $\approx 15$  Kcal mol $^{-1}$  K $^{-1}$ ),  
 112  $R_g$  is the gas constant (1.987 cal mol $^{-1}$ ), and  $T_0$  and  $T_1$  are in Kelvin for the  
 113 measured potential rate ( $\Phi$  or  $ETS_0$ ) and the predicted rate ( $R_1$ ), respec-  
 114 tively. It is important to note here that the Arrhenius Equation uses molar  
 115 units whereas the Boltzmann Factor in the MTE uses atomic units.  $S$  is the  
 116 reactant (substrate) of the enzyme reaction, controlling respiration.  $K$ , for  
 117 a single-reactant reaction ( $S \rightarrow P$ , where  $P$  is the product of the reaction),  
 118 is the Michaelis-Menten constant ( $K_m$ ).

119 In the case of a bisubstrate reaction, ( $S_1 + S_2 \rightarrow P_1 + P_2$ ),  $S$  becomes  
 120  $[S_1 S_2]$  and  $K$  becomes

$$K_\beta = K_{S_1} K_{ia} + K_{S_2} [S_1] + K_{S_1} [S_2] \quad (3)$$

121 where  $K_{ia}$  is the dissociation constant for the enzyme- $S_2$  complex (Packard  
 122 et al., 1996a,b, 2004). Note that  $A$ , the frequency factor in the Arrhenius  
 123 equation is eliminated algebraically because  $A$  is normally a constant for each  
 124 reaction. In the case of physiological processes (respiration, photosynthesis,  
 125 nitrogen fixation, etc.) we are assuming that  $A$  is a constant. Accordingly,  
 126  $A$  does not appear in Eq. (2). Furthermore if  $T_0$  and  $T_1$  are equal, as in this  
 127 paper, then  $e^{\frac{-E_a}{R_g(T_1-T_0)}}$  becomes 1 and Eq. (2) simplifies to:

$$R_0 = \frac{ETS_0 [S]}{K + [S]} \quad (4)$$

128 Note also the similarity between  $e^{\frac{-E_a}{R_g(T_1-T_0)}}$ , in Eq. (2), and  $e^{\frac{-E_a}{kT}}$ , in  
 129 Eq. (1). Both are derived from Maxwell's work in the 1850s and Boltz-  
 130 man's work in the 1860s, but the application to chemical rates was explained  
 131 by Arrhenius in 1889 and the application to biological rates was again the  
 132 work of Arrhenius around the turn of the century (Arrhenius, 1889, 1915).  
 133 Boltzmann explained the distribution of molecular velocities and from that  
 134 derived the perfect gas law. He did not explain the effect of temperature  
 135 on chemical or biological reactions. That was entirely the work of Svant  
 136 Arrhenius. Accordingly, an important difference between the temperature  
 137 functions in the EKM and the MTE is the use of  $R_g$  (from Arrhenius) in  
 138 EKM's Eq. (2) and  $k$  (from Boltzmann) in the MTE's Eq. (1). Numeri-  
 139 cally, with  $k$  in atomic units, the difference is enormous because  $k = \frac{R_g}{N}$  where  
 140  $N = \text{Avogadro's number}$ ,  $6.022 \times 10^{23} \text{ atoms mol}^{-1}$ . If  $k$  is in electron-volts,  
 141 it incorporates units that biologists, chemists, and biochemists rarely use and  
 142 even Richard Feynman, the Nobel laureate physicist, argued against using it  
 143 in the physics community (Feynman, 1998). Feynman thought it useful in

144 the atomic physics community, but not outside. For 100 years the biological-  
145 chemical community has been measuring and using energy of activations,  
146 gas constants, and the Arrhenius equation based on molar units, so a sudden  
147 switch to electron-volt units is a major and unnecessary change.

148 The predictive capability of the EKM for respiratory CO<sub>2</sub> production  
149 rates has been demonstrated in pyruvate-based cultures of the marine bac-  
150 terium, *Pseudomonas nautica* (Roy and Packard, 2001). In that experiment,  
151 measurements of isocitrate dehydrogenase activity, provided a proxy for po-  
152 tential respiratory CO<sub>2</sub> production. For respiratory oxygen consumption  
153 ( $R_{O_2}$ ), this model can predict rates in *P. nautica* from measurements of ETS,  
154 kinetic constants from the literature, and modelled time courses of the two  
155 main ETS electron donors (reactants), NADH and NADPH (Packard et al.,  
156 1996a). These reactants are represented by  $S_1$  and  $S_2$  in Eq. (3). Here we  
157 show in a feasibility study that this model (Eq. (5)) can be used to predict  
158  $R_{O_2}$  in a culture grown on an entirely different carbon source.

$$R_0 = \frac{ETS_0 [S_1 S_2]}{K_{S_1} K_{ia} + K_{S_2} [S_1] + K_{S_1} [S_2] + [S_1 S_2]} \quad (5)$$

159 The model works for an acetate-based, temperature-controlled culture  
160 of *Pseudomonas nautica* as well as for an acetate-based culture of another  
161 marine bacteria, *Vibrio natriegens*. The laboratory experiments show time-  
162 profiles of  $R_{O_2}$  and *in vitro* activity of the ETS throughout the exponential  
163 and stationary phases of both marine bacteria, *Pseudomonas nautica* and  
164 *Vibrio natriegens*. It demonstrates the difference in the relationships between  
165 the ETS and  $R_{O_2}$  in the exponential and stationary phases of the bacteria  
166 cultures. Finally it shows how respiratory control is achieved by substrate

167 modulation of the ETS. The model presented here demonstrates this sub-  
168 strate control. It is based on the concept that the concentration of the ETS  
169 substrates (NADH and NADPH) can be calculated from the concentration  
170 of the carbon source (acetate) in the culture medium and the biomass of the  
171 population. In addition, it is based on the assumption of bisubstrate kinetic  
172 control of the ETS activity in the bacteria populations. The model is tested  
173 by comparing its output, the respiration time-profile, with the measured  $R_{O_2}$   
174 time-profile in three experiments. Furthermore it is compared with the res-  
175 piration time-profile predicted by the MTE. We find that the MTE is not  
176 useful for predicting bacterial respiration beyond the exponential phase of  
177 growth. The EKM, on the other hand, predicts respiration in both the ex-  
178 ponential and the stationary phases.

179

## 180 2. Material and Methods

### 181 2.1. Bacterial cultures

182 *Pseudomonas nautica* (strain 617 from Dr. P Bonin, Université de la  
183 Méditerranée, Marseille, France) and *Vibrio natriegens* (ATCC 33788) were  
184 adapted to the acetate media for at least 15 generations prior to the experi-  
185 ments. Exponential or early stationary phase cultures were used to inoculate  
186 experiments. Cultures were continually shaken orbitally at 100 rpm at 22°C.  
187 Growth was monitored spectrophotometrically at 550 nm (OD550). Reagents  
188 for the culture media were obtained from Sigma. *Pseudomonas nautica*  
189 was cultured according to Packard et al. (1996a). The medium for *V. na-*  
190 *natriegens* was developed from the media of Niven et al. (1977); Baumann



191 and Baumann (1981); King and Berman (1984); Nissen et al. (1987) after  
192 experiments in the laboratory established the optimal growth conditions.  
193 It contained: 400 mM NaCl, 10 mM MgSO<sub>4</sub> 7H<sub>2</sub>O, 10 mM CaCl<sub>2</sub> 2H<sub>2</sub>O,  
194 10 mM KCl, 25 mM NH<sub>4</sub>Cl, 0.33 mM phosphate buffer, 0.01 mM FeSO<sub>4</sub> 7H<sub>2</sub>O,  
195 and 30 mM sodium acetate. All components (except FeSO<sub>4</sub> 7H<sub>2</sub>O and the  
196 phosphate buffer) were dissolved in 0.22  $\mu$ m filtered deionized water. The pH  
197 was adjusted to 7.5 with 1 N NaOH. The solution was filtered through a GF/F  
198 glass fiber filter to remove particles, and autoclaved for 45 min. at 121° C.  
199 To avoid precipitate formation during autoclaving, the phosphate buffer  
200 (0.67 M, pH 7.5) and FeSO<sub>4</sub> 7H<sub>2</sub>O solution (0.1 mM) were prepared sepa-  
201 rately. The phosphate buffer was autoclaved, but the iron sulphate solution  
202 was sterilized by filtration through 0.22  $\mu$ m acrodiscs. Both solutions were  
203 kept frozen and added to the culture medium on the day of use.

204 The basic experimental design was a time-course. Bacteria cultures were  
205 grown in 25 cotton-plugged 500 ml Erlenmeyer flasks containing 100 ml of  
206 media. Initial OD<sub>550</sub> after inoculation was 0.1. At about 2 hr intervals,  
207 2 flasks were chosen randomly, 25 ml of culture were transferred to the Oxy-  
208 max flasks, and the respiration was measured. Afterwards, the corresponding  
209 Erlenmeyer flasks were sampled for OD<sub>550</sub>, protein, ETS activity and acetate  
210 (in duplicate).

211 Respiration was measured in a Micro-Oxymax respirometer (Columbus  
212 Instruments International Corporation, Columbus, OH, USA) by measuring  
213 O<sub>2</sub> changes in the head space of the experimental flasks with an oxygen  
214 detector based on the principle of an PbO<sub>2</sub> fuel cell. The respirometer featured  
215 a multiple sample chamber (for up to 20 channels), a reference chamber, and

216 a computerized data acquisition and analysis system. A measurement was  
217 accomplished in 30 min. Aerobic conditions were assured because the Micro-  
218 Oxymax replenished the head-space air when O<sub>2</sub> levels fell below 19.3%.  
219 Respiration is reported as  $\mu\text{mol O}_2 \text{ min}^{-1} \text{ l}^{-1}$  (Fig. 1). The oxygen detector  
220 was calibrated with high precision gas standards. Each  $R_{O_2}$  measurement  
221 represents the mean of duplicate analyses. The range of the duplicates was  
222 10.0% of the mean (S.D. 9.4%, n=20).

223 For the acetate analysis 5 to 10 ml of culture were centrifuged at 10000  $\times$  g  
224 for 15 min at 4°C, the supernatant fluid was collected in an acid-rinsed  
225 Corex tube, and stored in liquid nitrogen. Later, samples were thawed and  
226 adjusted to pH 2 by adding 3  $\mu\text{l}$  of concentrated phosphoric acid. Acetate was  
227 detected in its acid form by high performance liquid chromatography (HPLC)  
228 system consisting of 2 pumps (Perkin-Elmer, Norwalk, CT, USA; Series 3B),  
229 a 20  $\mu\text{l}$  sample loop injector (Rheodyne, model 7125), a standard 4.6 mm  
230 I.D. reverse-phase C18 column (Supelcosil LC 18, d,<sub>r</sub>=3  $\mu\text{m}$ ), a precolumn  
231 (Supelcosil LC 18), and a UV-VIS variable wavelength detector (Perkin-  
232 Elmer LC-85 and the autocontrol module). The absorbance of acetate was  
233 detected at 210 nm. Sodium acetate (Sigma, more than 99% pure) served  
234 as the standard. Mobile phase was prepared using HPLC grade phosphoric  
235 acid and deionized water. All chromatographic measurements were carried  
236 out at 0.7 ml min<sup>-1</sup> using 0.05 M phosphoric acid as the mobile phase.

237 For the ETS activity measurements, 5 to 10 ml of culture (depending on  
238 the biomass) were centrifuged at 10000  $\times$  g for 15 min at 4°C. The pellets  
239 were stored in liquid nitrogen. Later they were resuspended in 2 ml of the  
240 homogenizing buffer at 0 to 4°C, and measured kinetically for ETS activity

241 with a modification of the Packard and Williams (1981) method. Details are  
242 given in Packard and Christensen (2004). Results are converted from ETS  
243 units of  $\mu\text{mol e}^- \text{min}^{-1} \text{l}^{-1}$  to potential respiration units in  $\mu\text{mol O}_2 \text{min}^{-1} \text{l}^{-1}$   
244 of culture by dividing by 4 ( $4\text{e}^- + 4\text{H}^+ + \text{O}_2 \rightarrow 2\text{H}_2\text{O}$ ).

245 For the protein analysis, pellet samples were taken, frozen, and stored as  
246 in the ETS analysis. The pellets were later resuspended in 2 to 4 ml 1 N NaOH  
247 (at 22°C) and mixed well. Protein analysis was performed on a 0.5 ml sample,  
248 using the method of Lowry et al. (1951). The homogenates were diluted if the  
249 absorbance at 750 nm exceeded 0.4, and analysed again. Bovine Serum Al-  
250 bumin (BSA) from Sigma Chemical Company was used as a standard. Mea-  
251 surements were made in duplicate. Their range around the average of these  
252 duplicates decreased from 14% during stationary (after 15 hr) to 2% during  
253 exponential growth. The mean of these ranges averaged 4.1%.

254 All measured time-courses of the culture biomass (protein), carbon source  
255 (acetate), potential respiration (*in vitro* ETS activity, (*ETS*)) and respira-  
256 tion presented here are listed in Tables 1, 2 and 3. The NADH and NADPH  
257 concentrations were calculated from the acetate and the protein as in Packard  
258 et al. (1996a), and as explained in the following section.

## 259 2.2. Respiration model

260 The conceptual idea of the model was originally developed from obser-  
261 vations of declining respiration soon after pyruvate declined in a pyruvate-  
262 limited batch culture of *Pseudomonas nautica* (Packard et al., 1996a). Here,  
263 one can see a similar situation with acetate. In Fig. 1 the time course of  $R_{\text{O}_2}$   
264 is characterized by low values of bacteria respiration after 20 hours during  
265 acetate limitation (stationary phase) at constant temperature. This suggests

266 that low levels of pyridine nucleotides (as ETS substrates), caused by low  
 267 levels of acetate, are throttling down the *in vivo* ETS activity from its  $V_{max}$   
 268 to a much lower rate, its actual respiration rate. Based on the observation  
 269 that the respiration falls in parallel with the falling levels of acetate, one  
 270 can intuit that respiration can be described, mathematically, by an enzyme  
 271 kinetic model where substrate-dependent enzyme reactions that control the  
 272 ETS activity play a key role (Eq. (6)). The basis of this EKM has been  
 273 explained in the *Introduction* here and in Packard et al. (1996a); Roy and  
 274 Packard (2001); Packard et al. (2004); Packard and Gómez (2008).

275 Here, this type of model has been applied to temperature-controlled cul-  
 276 tures of *P. nautica* and *V. natriegens* to test its ability to predict respiratory  
 277 oxygen consumption ( $R_m$ ) in both the exponentially growing phase and the  
 278 nutrient-limited stationary phase. The model is based on the following equa-  
 279 tions:

$$R_m = \frac{ETS [NADH] [NADPH]}{K_\beta + [NADH] [NADPH]} \quad (6)$$

280 where

$$K_\beta = K_{NADH} K_{ia} + K_{NADPH} [NADH] + K_{NADH} [NADPH] \quad (7)$$

281 This is the equation for a bisubstrate enzyme controlled reaction (Segel,  
 282 1993). The concentration of the ETS substrates (NADH and NADPH) were  
 283 modelled from the cell protein and the acetate concentration in the culture  
 284 medium, both of the previous hour. Mathematically this means that the  
 285 value of each of the pyridine nucleotides at any time was calculated from the

286 time-averaged values of acetate ( $P$ ) and cell protein ( $M$ ) (Eqs. (8) and (9))  
287 over the previous sampling period.

$$NADH = \delta P + \omega M \quad (8)$$

$$NADPH = \lambda P + \eta M \quad (9)$$

288 This use of a lag function in the calculations is an attempt to incorpo-  
289 rate the role of cell history in determining metabolism (Roy and Packard,  
290 2001). Accordingly, the intracellular NADP and NADPH time profiles were  
291 modelled as functions of the mean extracellular  $P$  and  $M$  (Eqs. (8) and (9))  
292 during the previous intersampling period. The calculations of these mid-  
293 point values are shown in Tables 1, 2 and 3. All the equations used in the  
294 EKM are summarized in Table 4.

295 To evaluate the models,  $R_{O_2}$  for each experiment (Fig. 1) was modelled  
296 using the MTE and the EKM and contrasted with the measured time-profile  
297 of respiration. This way, each model's efficiency in reproducing the bacterial  
298 respiration time-course in the two different physiological states would be seen  
299 clearly. To make these calculations for a constant temperature with the MTE  
300 one needs to reduce Eq. (1) to:

$$R = C M^b \quad (10)$$

301 The data for  $M$  are listed in Tables 1, 2 and 3.

302 Note that, at a constant temperature, the Boltzmann factor in Eq. (1)  
303 is no longer involved. The values  $C$  and  $b$ , in Eq. (10), represent the stoi-  
304 chiometric factor or 'normalization constant' (Brown et al., 2004; Allen and

305 Gillooly, 2007) and Kleiber’s Law scaling factor, respectively. For the value  
 306 of  $b$ , one can use  $3/4$  from Kleiber’s Law or one can determine it from the  
 307 experimental data. Here, we have used the data from the exponential growth  
 308 phase of the culture with *P. nautica* (Experiment A) (Table 1) and the cul-  
 309 ture with *V. natriegens* (Experiment B) (Table 2), and plotted  $\text{Log}_{10} R$   
 310 versus  $\text{Log}_{10} M$ . By this analysis  $C$  is equal to the antilogarithm of the in-  
 311 tercept and  $b$  is the slope of the regression line. This procedure insures that  
 312 the MTE can make its best prediction; if all the data had been used there  
 313 would have been no useful relationship between  $R$  and  $M$  and the MTE pre-  
 314 diction would have been worse. Accordingly, by using only the exponential  
 315 phase data to calculate those expressions, the  $r^2$  was 0.986 and 0.999 for Ex-  
 316 periment A and Experiment B, respectively. Because Experiment C was a  
 317 long-term study it had only three data points within the exponential growth  
 318 phase (Table 3). Using these three points would not have yielded a reliable  
 319 algorithm. Consequently, we have used the expression from Experiment B  
 320 (Eq. 12), which by having seven data points for the same species and carbon  
 321 source yielded a more robust equation. The resulting MTE algorithms for  
 322 the three experiments at constant temperature became:

$$R = 1.0359 M^{0.7963} \quad (\textit{Experiment A}) \quad (11)$$

$$R = 0.7671 M^{0.7795} \quad (\textit{Experiment B and C}) \quad (12)$$

323 The comparable calculation with the EKM at a constant temperature  
 324 was made with Eq. (6) and Eq. (7).

325 *2.3. Modeling computation*

326 The initial calculations for this acetate-based model used the pyruvate-  
327 based model of the marine bacterium *P. nautica* (Packard et al., 1996a) for  
328 all three experiments. Accordingly, for the ETS-substrates, NADPH and  
329 NADH, we used the same algorithms (Eqs. (8) and (9)) as in Packard et al.  
330 (1996a) with the same parameters (Table 5, *Column 1*). In all cases the  
331 input consisted of smoothed time-course data following the Loess method  
332 (Hutcheson, 1995). In optimizing the model for experiments with *Vibrio*  
333 *natriegens*, we changed the parameters  $\lambda$  and  $\delta$ . In VnAc1105, the parameters  
334  $\lambda$  and  $\delta$  were reduced by a third using a factor of 0.3294 (Table 5, *Column 2*).  
335 In VnAc2601 the same two parameters,  $\lambda$  and  $\delta$ , were doubled using a factor  
336 of 2.1782 (Table 5, *Column 3*).

337 The acetate-dependent part of the equations for NADH and NADPH  
338 serves as the ‘substrate throttle’. The cell-protein (biomass)-dependent part  
339 of these equations serves as a base-line. To calculate this second part, we  
340 assumed the pyridine nucleotide ratio to cell-protein to be the same in these  
341 acetate-based cultures as it was in the pyruvate-based ones of Packard et al.  
342 (1996a). In this way we were able to keep the same parameters  $\omega$  and  $\eta$   
343 (Eqs. (8) and (9)) as previously used (Table 5).

344 The optimization of the parameters was done assuming the decrease of  
345 the ETS-substrates, NADH and NADPH, occurs in parallel as was predicted  
346 in Packard et al. (1996a). Hence, one can use the same factor to correct  
347 simultaneously both substrate-throttle parameters,  $\lambda$  and  $\delta$ . Consequently,  
348 a loop that searched for the optimum correction factor was computed by  
349 calculating the NADH and NADPH time-courses that best predicted the

350 oxygen consumption during the experiment. This technique estimated the  
351 output respiration from the EKM (Eq. (6)) by looking for the parameter  
352 that provides a linear regression model with a slope close to 1. It used  
353 the smoothed time-profile of  $R_{O_2}$  as the standard. This technique forced  
354 the model towards the most realistic prediction as possible. The reliability  
355 of the parameters found for the marine bacterium *Vibrio natriegens* was  
356 judged on two criteria. First, their ability to generate declining time profiles  
357 of NADH and NADPH within a biologically reasonable range (White et al.,  
358 1964; Lehninger, 1970; Walsh and Koshland Jr., 1984; Lehninger et al., 1993)  
359 as the acetate diminished in the culture media. Second, their ability to  
360 provide realistic respiration output data throughout the time-course of the  
361 experiment. For the kinetic constants ( $K_{ia}$ ,  $K_{NADH}$  and  $K_{NADPH}$  in Table 2)  
362 we used those from Experiment B in Packard et al. (1996a).

### 363 **3. Results and Discussion**

364 Fig. (1) shows time-courses of the culture biomass (cell-protein), carbon  
365 source (acetate), potential respiration (the *in vitro* ETS,  $A_{ETS}$ ) and measured  
366 respiration for one experiment with *P. nautica* and two experiments with *V.*  
367 *natriegens*. In the beginning the cultures grew exponentially on the acetate;  
368 they passed through a short stationary phase; and then, as the acetate was  
369 exhausted, they fell into a senescent state. The experiments used this tran-  
370 sition between exponential growth and senescence to separate the enzymatic  
371 capacity for respiration, the ETS activity, from the physiological expression  
372 of this capacity, the measured oxygen consumption. This strategy enabled  
373 us to challenge the predictive capability of the two models. It facilitated



374 the comparison of the EKM's output to the MTE's output during realistic  
375 biological conditions. During the exponential growth phase, prediction from  
376 either ETS activity or cell protein is not a challenge because respiration,  
377 ETS activity and cell-protein trend in parallel. However, during senescence  
378 cell-protein and ETS activity trend together, but respiration breaks away  
379 and decreases rapidly. Thus in senescent phase predicting respiration from  
380 either cell-protein or ETS activity requires causal-level understanding of the  
381 respiratory mechanism.

382 Acetate was consumed rapidly as the respiration, ETS activity, and cell-  
383 protein increased. All cultures behaved similarly (Fig. 1). Shortly after the  
384 acetate was exhausted the respiration declined to very low levels even though  
385 the ETS activity and the bacterial biomass remained high. In this situation,  
386 with the carbon source exhausted, the respiration appears uncoupled from  
387 both the biomass and the ETS activity. In effect, within 7 hours the ratio of  
388 both the respiration to cell-protein and the ratio of respiration to ETS activ-  
389 ity decreased to 0.2 and 0.25 of their value during exponential phase. This  
390 can explain some of the error in respiration inherent in both Kleiber's law  
391 and the R/ETS ratio used in oceanographic research. What is the cause of  
392 this apparent uncoupling if the ETS is the causal basis of respiration? The  
393 parallelism in the declining acetate and declining respiration rate (Fig. 1)  
394 provides a clue. If the substrates for the electron transport complexes fall  
395 as does the acetate, then the activity of these enzyme complexes would be  
396 throttled down the way the reaction rate in an enzyme-catalyzed reaction is  
397 modulated by substrate levels in a Michaelis-Menten equation (Fig. 2). Here,  
398 these substrate declines were modelled from the acetate declines in the three

399 experiments (Fig. 1) using Eqs. (8) and (9). At this point it must be remem-  
400 bered that the ETS activity measured at any time in the bacteria cultures,  
401 whether the cells are in exponential or in senescent growth, is the activity  
402 measured in the presence of unlimited substrates. This condition forces the  
403 ETS complexes to react with NADH and NADPH at the complexes' maxi-  
404 mum capacity regardless of how fast they were reacting in the living, intact  
405 bacteria cell. Thus ETS in Eq. (6) is equivalent to a Michaelis-Menten  $V_{max}$   
406 as we have said in the *Introduction*.

407 We emphasize that the EKM is based on the observation that bacte-  
408 rial respiration declines in parallel with declining concentrations of carbon  
409 source (acetate) in the culture medium suggesting that natural ETS sub-  
410 strates would also decline in parallel with the carbon source. Accordingly  
411 a rectangular hyperbola from the Michaelis-Menten expression, describing a  
412 declining reaction rate as a function of falling substrate, explains the decrease  
413 in the *in vivo* ETS activity and hence the whole-cell respiration rate. Note  
414 here, that the *in vivo* ETS activity is the unmeasured ETS activity in the  
415 cell, not the ETS activity measured in a test tube. This later ETS activity  
416 (*in vitro*) is the Michaelis-Menten  $V_{max}$  discussed above. The former ETS  
417 activity in the cell is equivalent to the whole-cell respiration rate. Our entire  
418 effort is an attempt to create a conceptual and mathematical bridge between  
419 the later and the former ETS activities. This approach, as embodied in our  
420 previous model (Packard et al., 1996a), successfully predicted respiration in  
421 pyruvate-based cultures of *Ps. nautica*. Here, the same model predicts the  
422 respiration in acetate-based cultures of both *P. nautica* and *V. natriegens*  
423 (Fig. 3). The three respiration predictions from the original model are good,

424 especially for *P. nautica* (Fig. 4, Panel A), but because the predictions for  
425 *V. natriegens* (Fig. 4, Panels B and C) were not optimum, parameters  $\lambda$   
426 and  $\delta$  were modified again to produce the new pyridine nucleotide profiles  
427 in Fig. 5 (Panels A and B) and new respiration predictions. These new  
428 respiration predictions, as well as a replot of the original respiration predic-  
429 tion for the experiment with *P. nautica* are shown in Panel A of Figs. 6, 7  
430 and 8. They do improve the prediction of respiration in experiments with  
431 *V. natriegens*. It now falls on future laboratory measurements of the actual  
432 NADH and NADPH time courses to verify both the concept of the EKM and  
433 the parameters  $\lambda$  and  $\delta$ .

434 In order to show the predictive capacity of the two respiration models  
435 (EKM and MTE) in the different physiological phases of bacterial growth,  
436 the modelled respiration and measured respiration are compared in Panels A-  
437 B and C-D, respectively, of Figs. 6, 7 and 8. These plots consider all the data  
438 from the beginning of the three cultures to their ends, so all physiological  
439 states are considered. The coefficients of determination,  $r^2$  values, for the  
440 EKM are all above 0.94 while for the MTE they would be meaningless and  
441 so they were not calculated. In effect, the respiration time-courses predicted  
442 by the MTE in Panel C of Figs. 6, 7 and 8 completely misrepresent the  
443 measured respiration time-course during steady state and nutrient limitation  
444 conditions. The MTE only models respiration well during the exponential  
445 growth phase. In contrast, the EKM predicts the respiration all through the  
446 different phases of bacterial growth.

447 **4. Conclusions**

448 Respiratory oxygen consumption in two species of marine bacteria, during  
449 exponential growth, steady state and nutrient-limited stationary phases, can  
450 be modelled from measurements of the *in vitro* respiratory electron transport  
451 system activity (ETS), the cell protein, the carbon source (acetate). The  
452 model's algorithm is based on Michaelis-Menten substrate kinetics. If the  
453 predicted NADH and NADPH time courses are verified, future respiration  
454 calculations will be made solely from measurements of ETS activity, [NADH]  
455 and [NADPH] via Eq. (6).

456 This Enzyme Kinetic Model, besides having a better mechanistic basis,  
457 describes respiration better than does the Metabolic Theory of Ecology model  
458 under conditions of nutrient-limitation.

459 We argue that respiration modeling could be improved by recognizing  
460 that the respiratory electron transport system, and not biomass, is the causal  
461 base of respiration, that the ETS is regulated by the availability of reduced  
462 pyridine nucleotides, and that it responds to temperature changes via the  
463 impact of temperature on the Arrhenius energy of activation as described by  
464 the Arrhenius equation.

465 The model we propose is expressed as:

$$R_1 = \frac{ETS_0 [S] e^{\frac{-E_a}{R_g(T_1 - T_0)}}}{K + [S]} \quad (2)$$

466 where  $R_1$  is the respiration rate measured at  $T_1$ ,  $ETS_0$  is the potential  
467 respiration rate (the Michaelis-Menten  $V_{max}$  of the ETS), measured at an-  
468 other temperature ( $T_0$ ),  $K$  is a bisubstrate kinetics expression analogous to  
469 the Michaelis-Menten  $K_m$ , and S represents the substrates (see *Introduction*).

470 In this model when  $T_0 = T_1$  the model equation reduces to:

471

$$R_0 = \frac{ETS_0 [S]}{K + [S]} \quad (4)$$

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576 **List of Captions (Tables and Figures)**

577

578 Table 1. *Pseudomonas nautica*. Data from Experiment A (PnAc290693). Mid-Acetate  
579 and Mid-Protein are the time-averaged values of acetate ( $P$ ) and cell protein ( $M$ ) over the  
580 previous sampling period. These mid-points are used to model the intracellular NADP and  
581 NADPH time profiles for Eqs. (8) and (9). \* The acetate beyond the lowest value (0,82)  
582 increased slowly to a value of 2,04, but for analytical reasons was considered unreliable.  
583 Consequently for modeling we assigned a value of 0,01.

584

585 Table 2. *Vibrio natriegens*. Data from Experiment B (VnAc110593). Mid-Acetate  
586 and Mid-Protein are the time-averaged values of acetate ( $P$ ) and cell protein ( $M$ ) over the  
587 previous sampling period. These mid-points are used to model the intracellular NADP  
588 and NADPH time profiles for Eqs. (8) and (9). \* The acetate beyond the lowest value (0)  
589 was not detectable, for modeling we assigned a value of 0,01.

590

591 Table 3. *Vibrio natriegens*. Data from Experiment C (VnAc260193). Mid-Acetate  
592 and Mid-Protein are the time-averaged values of acetate ( $P$ ) and cell protein ( $M$ ) over the  
593 previous sampling period. These mid-points are used to model the intracellular NADP  
594 and NADPH time profiles for Eqs. (8) and (9). \* The acetate beyond the lowest value (0)  
595 was not detectable, for modeling we assigned a value of 0,01.

596

597 Table 4. Summary of equations used in the Enzyme Kinetic Model (EKM).

598

599 Table 5. Kinetic constants and parameters that were used to model (EKM) the in-  
600 tracellular NADH and NADPH concentrations from Eqs. (8) and (9) and to predict the  
601 respiration rate from Eqs. (6) and (7). The units for the kinetic constants are  $\mu M$ . The  
602 units for the parameters are as follows:  $\lambda$ ,  $\mu mol$  NADPH (mmol pyruvate) $^{-1}$ ;  $\eta$ ,  $\mu mol$   
603 NADPH (mg protein) $^{-1}$ ;  $\delta$ ,  $\mu mol$  NADH (mmol pyruvate) $^{-1}$ ; and  $\omega$ ,  $\mu mol$  NADPH (mg  
604 protein) $^{-1}$ . *Column 1* lists kinetic constants and parameters used in Experiment B of  
605 Packard et al. (1996a) and in all three experiments of this work. All the values of  $\lambda$  and  $\delta$

606 used in this work are numerically the same as in Packard et al. (1996a) but for acetate. The  
607 results of their used are shown in Fig. 2. *Column 2* and *Column 3* list kinetic constants  
608 and parameters (revised  $\lambda$  and  $\delta$ ) used for Experiment B and Experiment C, respectively,  
609 in this work. The results of their use are shown in Fig. 5. Parameters in *Column 2* and 3  
610 have been changed as described in the text.

611

Figure 1: Original data (symbols) and interpreted (Loess method (Hutcheson, 1995)) time-course data (lines) for three experiments with two species of marine bacteria growing in acetate-based batch cultures. Panel A: *Pseudomonas nautica* in Experiment A showing observations of ETS activity (*in vitro*),  $R_{O_2}$ , protein and acetate over 27h. Panel B: *Vibrio natriegens* in Experiment B showing the same observations as in Panel A (same legend) but over 34h. Panel C: *Vibrio natriegens* in Experiment C as in Panel A and B but for 500h. The physiological state of the cells shifts between hours 10 and 20 from a well-nourished condition to a nutrient-limited condition. Analytical errors are given in *Material and Methods*.

Figure 2: Simulated time-courses for intracellular NADH and NADPH as calculated from (Eqs. (8) and (9)) for each of the three experiments. Panel A: *Pseudomonas nautica* in Experiment A. Panel B: *Vibrio natriegens* in Experiment B. Panel C: *Vibrio natriegens* in Experiment C. These time-courses used the kinetic constants and parameters listed in Table 5 (*Column 1*) and served as input in modeling the respiration in Fig. 3.

Figure 3: EKM modeling results using the original model from Packard et al. (1996a), but with acetate-based cultures instead of pyruvate-based ones. Panel A: *Pseudomonas nautica*. Experiment A showing the time-courses of measured respiration and *in vitro* ETS and compared to the modelled (EKM) respiration (from Eq. (6)). Panel B: *Vibrio natriegens*. Experiment B as in Panel A. Panel C: *Vibrio natriegens*. Experiment C as in Panel A and B.

Figure 4: Comparing modelled (Fig. 3) and measured (Fig. 1) respiration in all three experiments. In the linear regression equations the slope indicates the accuracy of the model. The coefficient of determination ( $r^2$  values) indicates the fidelity of the modelled respiration to the shape of the measured respiration. Panel A: *Pseudomonas nautica*. Experiment A. Panel B: *Vibrio natriegens*. Experiment B as in Panel A. Panel C: *Vibrio natriegens*. Experiment C as in Panel A and B.

Figure 5: Simulated time-courses for intracellular NADH and NADPH, as before, but recalculated from (Eqs. (8) and (9)). Panel A: *Vibrio natriegens* in Experiment B. Panel B: *Vibrio natriegens* in Experiment C. These time-courses used the kinetic constants and revised parameters listed in Table 5 (*Column 2* and *Column 3*) and served as input for the revised respiration models in Panel A of Figs. 7 and 8.

Figure 6: *Pseudomonas nautica* (Experiment A). Panel A: EKM modeling results as in Fig. 3 using the NADH and NADPH time courses shown in Panel B of Fig. 2. Panel B: Comparison between the measured and EKM modelled respiration shown in Panel A. Panel C: MTE modeling results based on measured cell-protein (M) and Eq. (11). Panel D: Comparison of measured and modelled respiration from the MTE.

Figure 7: *Vibrio natriegens* (Experiment B). Panel A: EKM modeling results as in Fig. 3, but using the NADH and NADPH time courses shown in Panel A of Fig. 5. Panel B: Comparison between the measured and EKM modelled respiration shown in Panel A. Panel C: MTE modeling results based on measured cell-protein (M) and Eq. (12). Panel D: Comparison of measured and modelled respiration from the MTE.

Figure 8: *Vibrio natriegens* (Experiment C). Panel A: EKM modeling results as in Fig. 3, but using the NADH and NADPH time courses shown in Panel B of Fig. 5. Panel B: Comparison between the measured and EKM modelled respiration shown in Panel A. Panel C: MTE modeling results based on measured cell-protein (M) for *Vibrio natriegens* in Experiment B and Eq. (12). Panel D: Comparison of measured and modelled respiration from the MTE.

Table 1:

Time	Acetate	Protein	ETS	$R_{O_2}$	Mid-Acetate	Mid-Protein
hours	mM	mg l <sup>-1</sup>	$\mu\text{mol O}_2 \text{ min}^{-1} \text{ l}^{-1}$	$\mu\text{mol O}_2 \text{ min}^{-1} \text{ l}^{-1}$	mM	mg l <sup>-1</sup>
0	31,59	12,83	2,95	-	31,59	12,83
2,67	31,79	19,49	1,80	6,10	31,69	16,16
5,50	28,41	35,14	26,99	14,08	30,10	27,31
7,83	24,18	76,65	37,77	33,19	26,29	55,89
9,08	21,08	123,07	87,17	47,13	22,63	99,86
11,50	15,88	177,56	121,95	52,90	18,48	150,32
13,83	5,37	282,49	216,00	47,32	10,63	230,03
16,75	0,82	322,06	283,58	46,06	3,10	302,27
17,83	*	372,28	224,62	27,77	0,41	347,17
19,00	*	335,81	195,06	5,60	0,01	354,04
21,75	*	331,88	266,55	2,01	0,01	333,84
24,83	*	344,50	274,70	1,13	0,01	338,19
27,67	*	338,89	203,49	0,90	0,01	341,70

Table 2:

Time	Acetate	Protein	ETS	$R_{O_2}$	Mid-Acetate	Mid-Protein
hours	mM	mg l <sup>-1</sup>	$\mu\text{mol O}_2 \text{ min}^{-1} \text{ l}^{-1}$	$\mu\text{mol O}_2 \text{ min}^{-1} \text{ l}^{-1}$	mM	mg l <sup>-1</sup>
0	47,00	10,26	2,37	-	47,00	10,26
3,00	46,65	26,44	17,56	5,37	46,83	18,35
4,83	42,60	43,73	50,40	12,66	44,63	35,09
5,83	42,40	63,98	39,54	15,15	42,50	53,86
6,83	40,20	81,36	50,72	22,80	41,30	72,67
8,17	34,60	156,41	122,11	31,70	37,40	118,89
9,25	29,20	157,78	145,95	42,90	31,90	157,10
10,25	25,75	206,67	147,84	45,03	27,48	182,23
11,25	20,75	222,46	176,97	44,83	23,25	214,57
14,50	8,10	268,08	193,34	45,30	14,43	245,27
18,75	0*	297,54	199,88	13,05	4,06	282,81
19,75	-*	291,65	202,76	8,14	0,01	294,60
22,50	-*	334,55	217,48	6,21	0,01	313,10
34,50	-*	312,97	149,87	1,83	0,01	323,76

Table 3:

Time	Acetate	Protein	ETS	$R_{O_2}$	Mid-Acetate	Mid-Protein
hours	mM	mg l <sup>-1</sup>	$\mu\text{mol O}_2 \text{ min}^{-1} \text{ l}^{-1}$	$\mu\text{mol O}_2 \text{ min}^{-1} \text{ l}^{-1}$	mM	mg l <sup>-1</sup>
0	35,90	5,60	1,30	0,64	35,90	5,60
17,50	28,67	73,76	17,07	14,62	32,29	39,68
40,50	0*	156,70	24,74	13,24	14,34	115,23
64,00	-*	156,60	27,37	2,67	0,01	156,65
138,50	-*	141,69	23,71	0,50	0,01	149,15
232,50	-*	126,46	16,80	0,33	0,01	134,08
496,50	-*	107,52	6,18	0	0,01	117,00



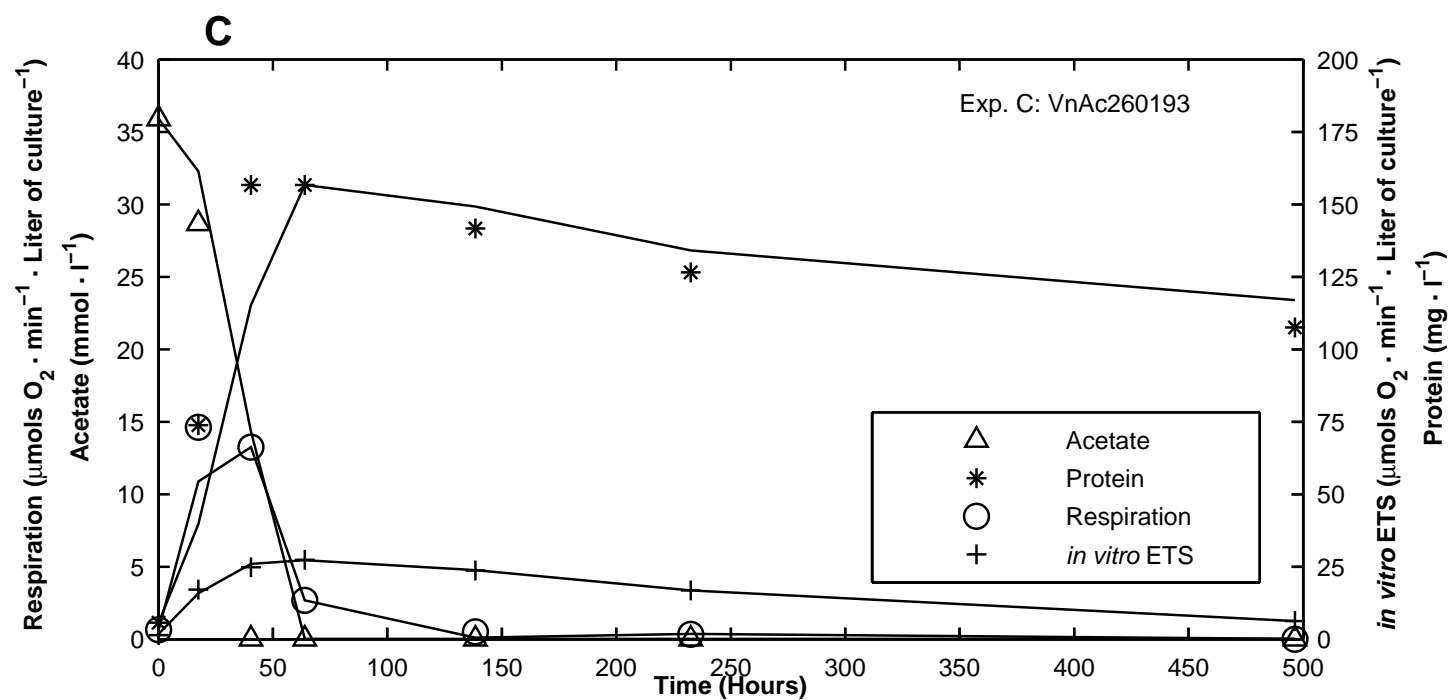
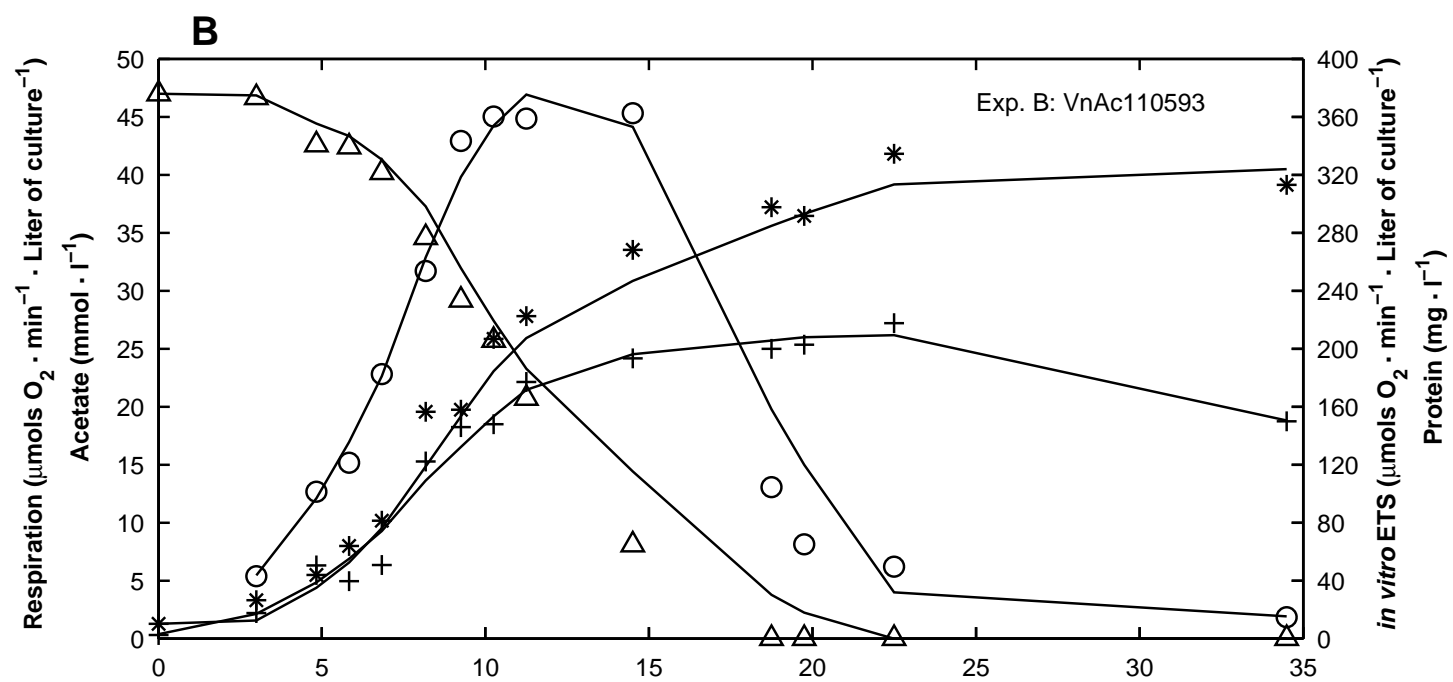
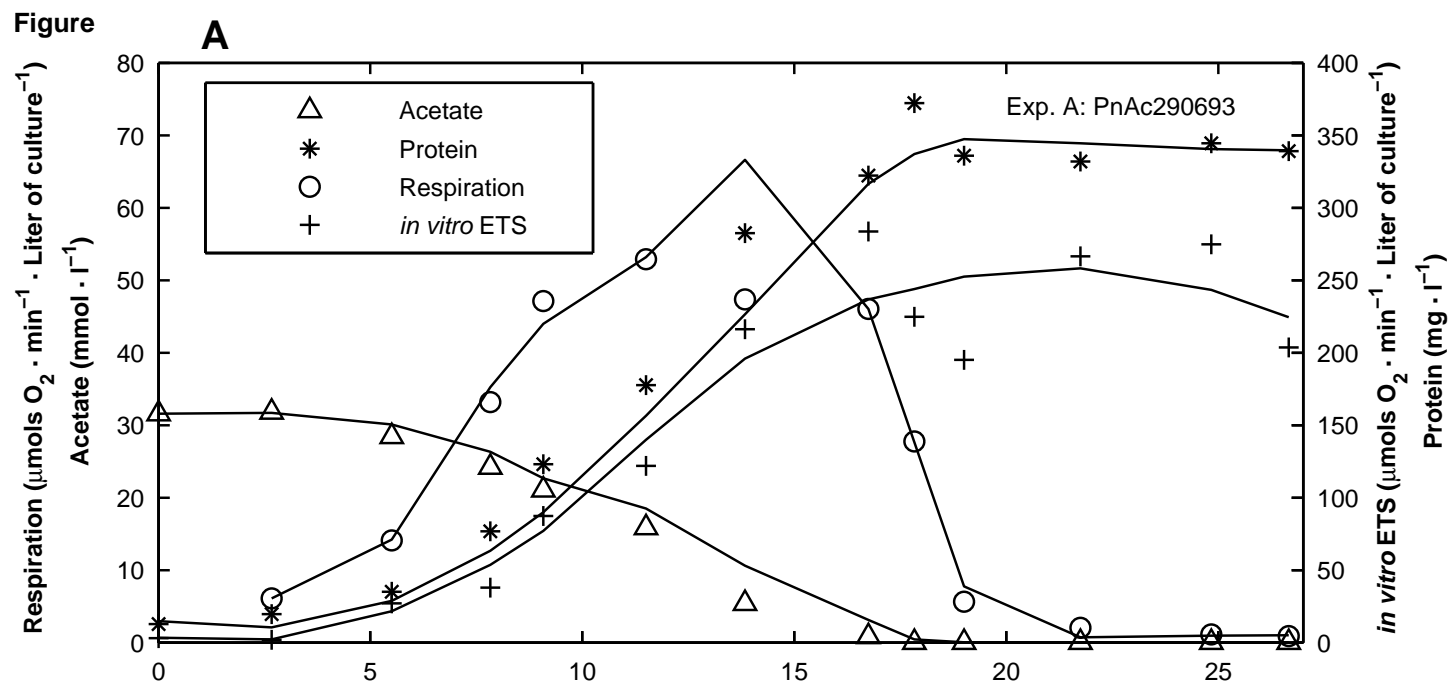
Table 4:

Eq.	N°
$R_m = ETS [NADH] [NADPH] / (K_\beta + [NADH] [NADPH])$	(6)
$K_\beta = (K_{NADH})(K_{ia}) + (K_{NADPH})[NADH] + (K_{NADH})[NADPH]$	(7)
$NADH = \delta P + \omega M$	(8)
$NADPH = \lambda P + \eta M$	(9)

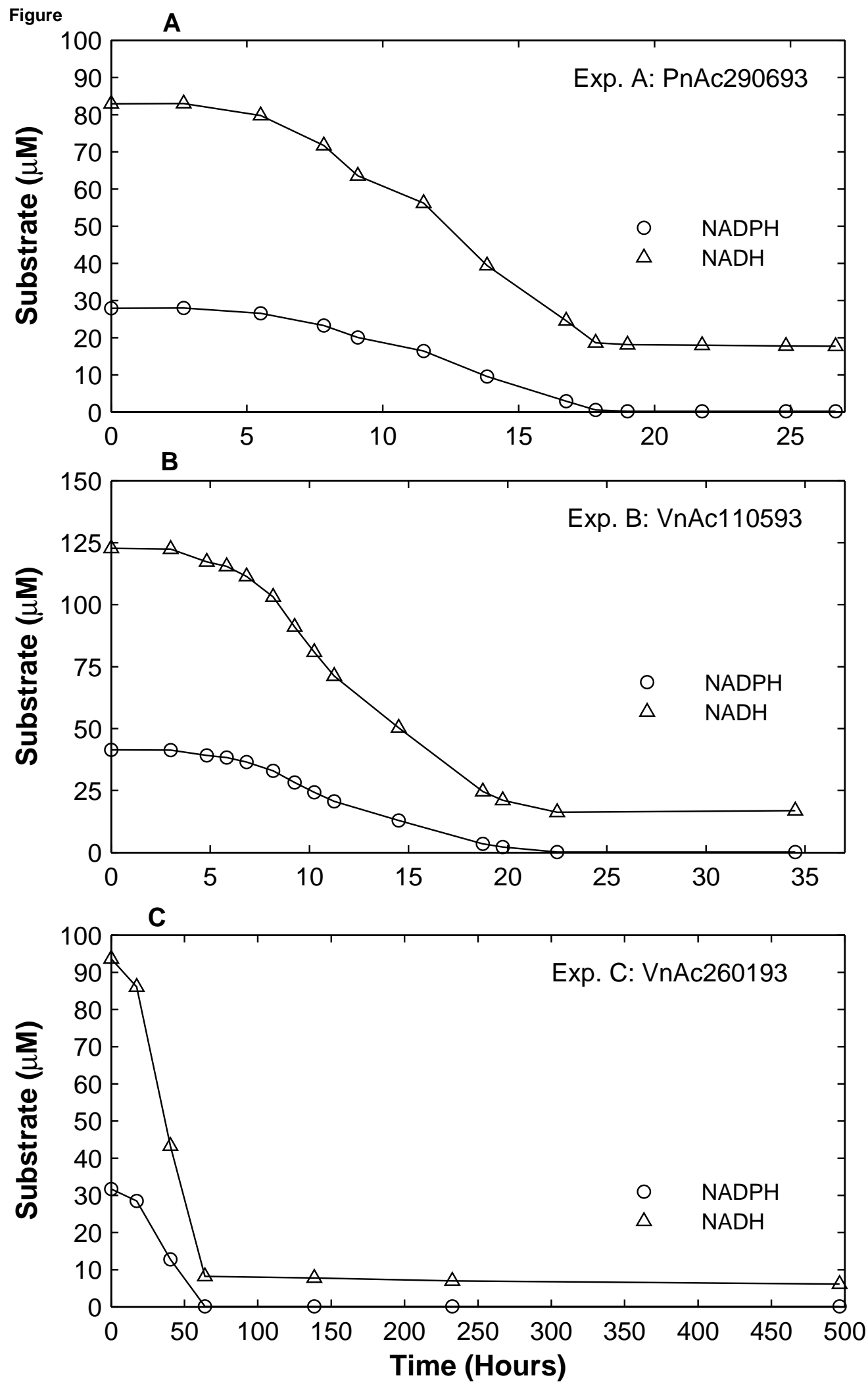
Table 5:

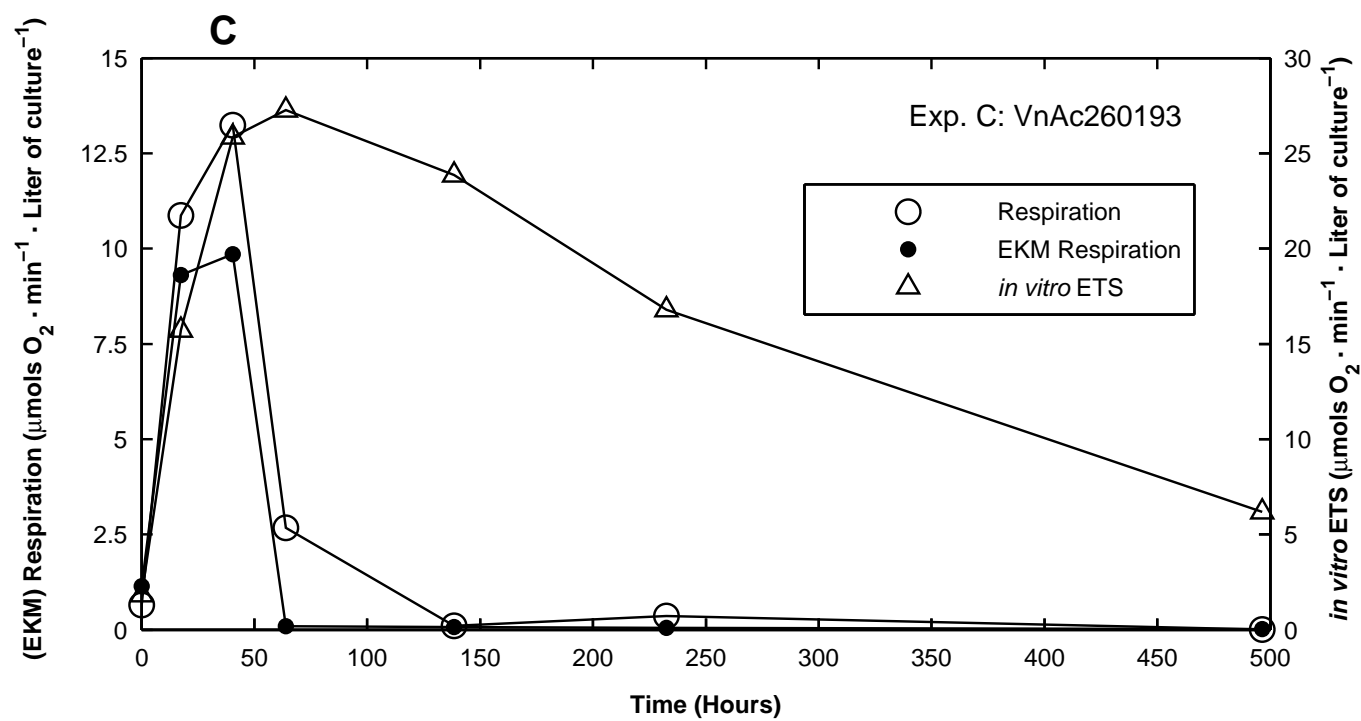
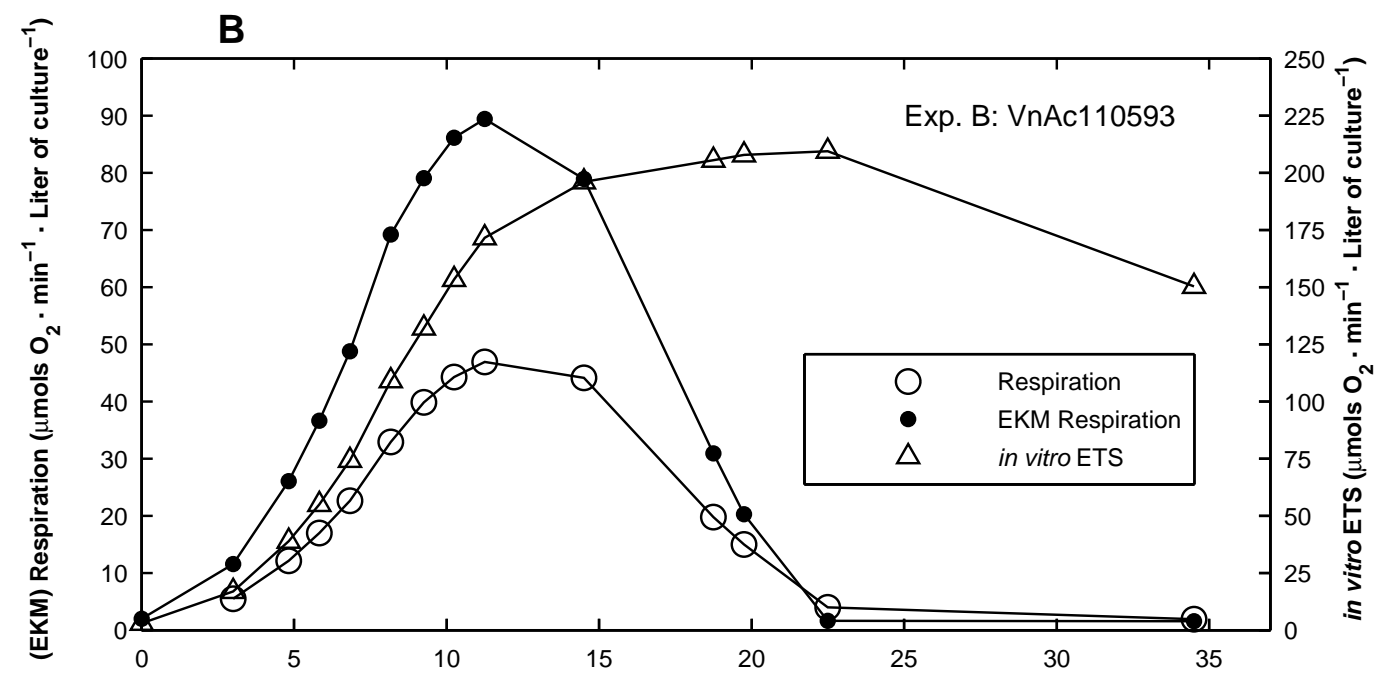
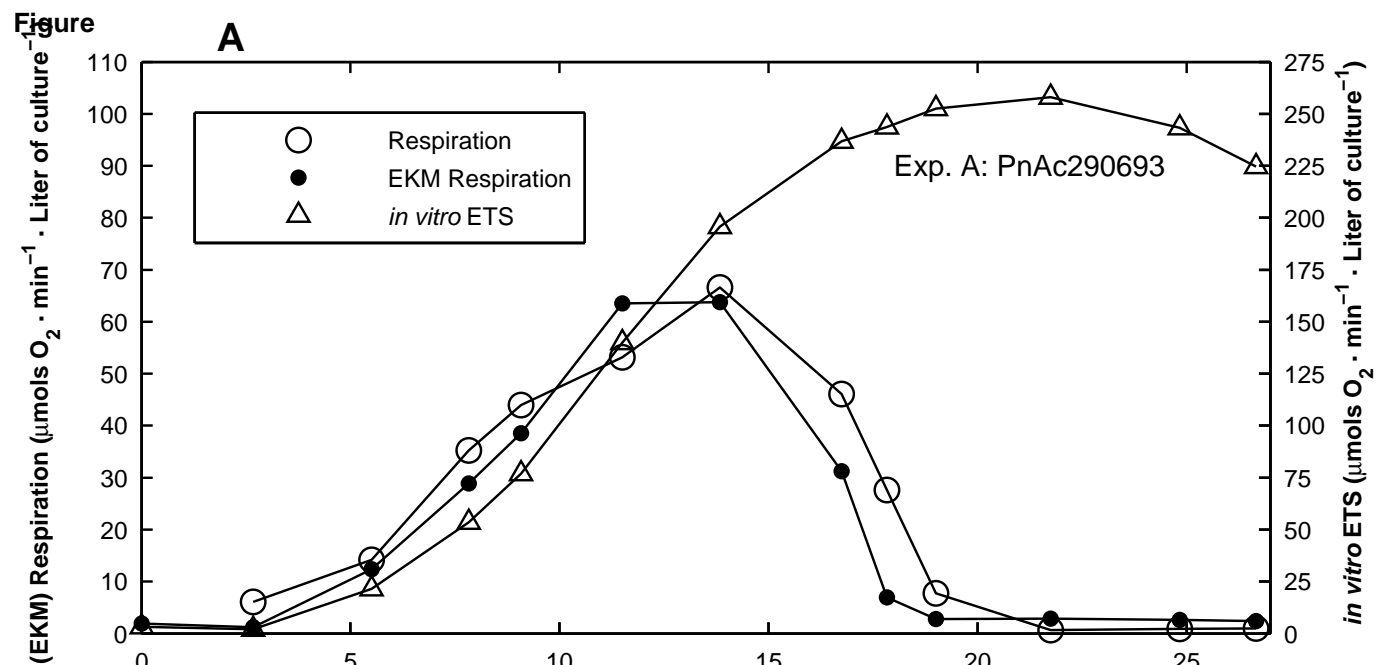
	Packard et al 1996	This work (Exp. B)	This work (Exp. C)
For Eqs. (6) and (7)			
$K_{ia}$	6.7	Same	Same
$K_{NADPH}$	9.0	Same	Same
$K_{NADH}$	26.0	Same	Same
For Eqs. (8) and (9)			
$\lambda$	0.882	0.3014	1.392
$\eta$	$5.90 \times 10^{-4}$	Same	Same
$\delta$	2.60	0.8884	4.1046
$\omega$	$5.20 \times 10^{-2}$	Same	Same

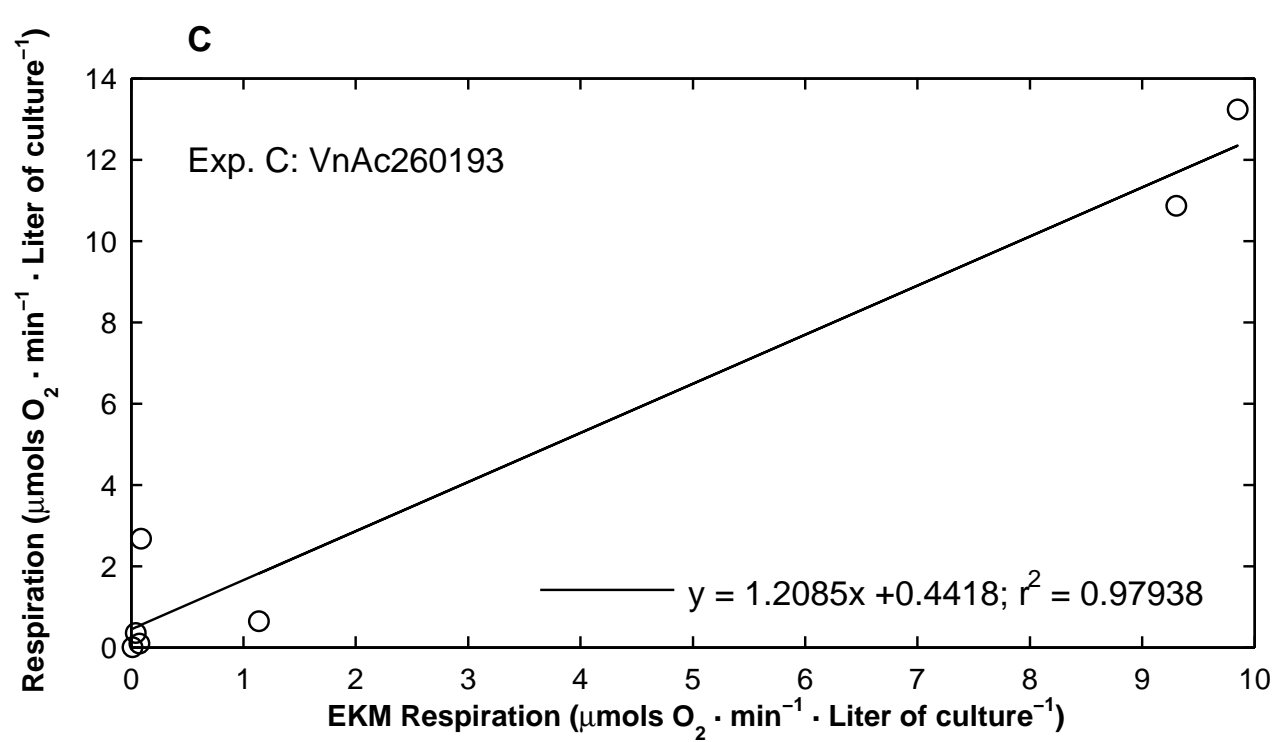
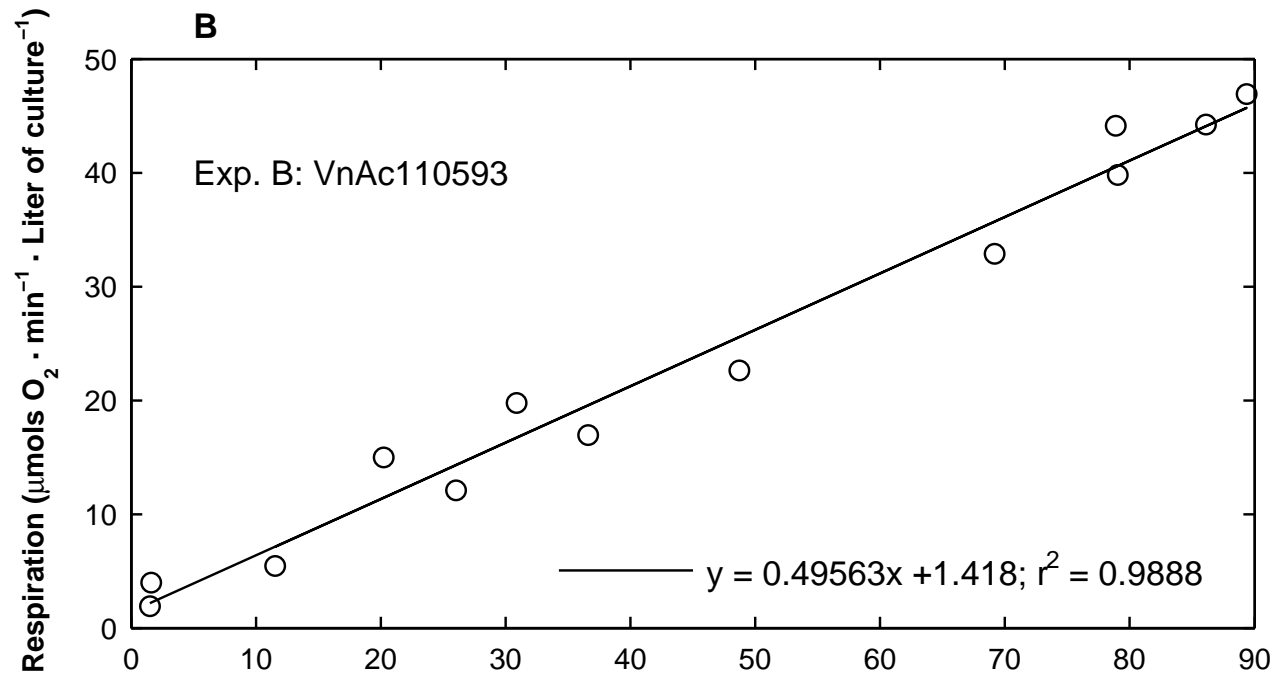
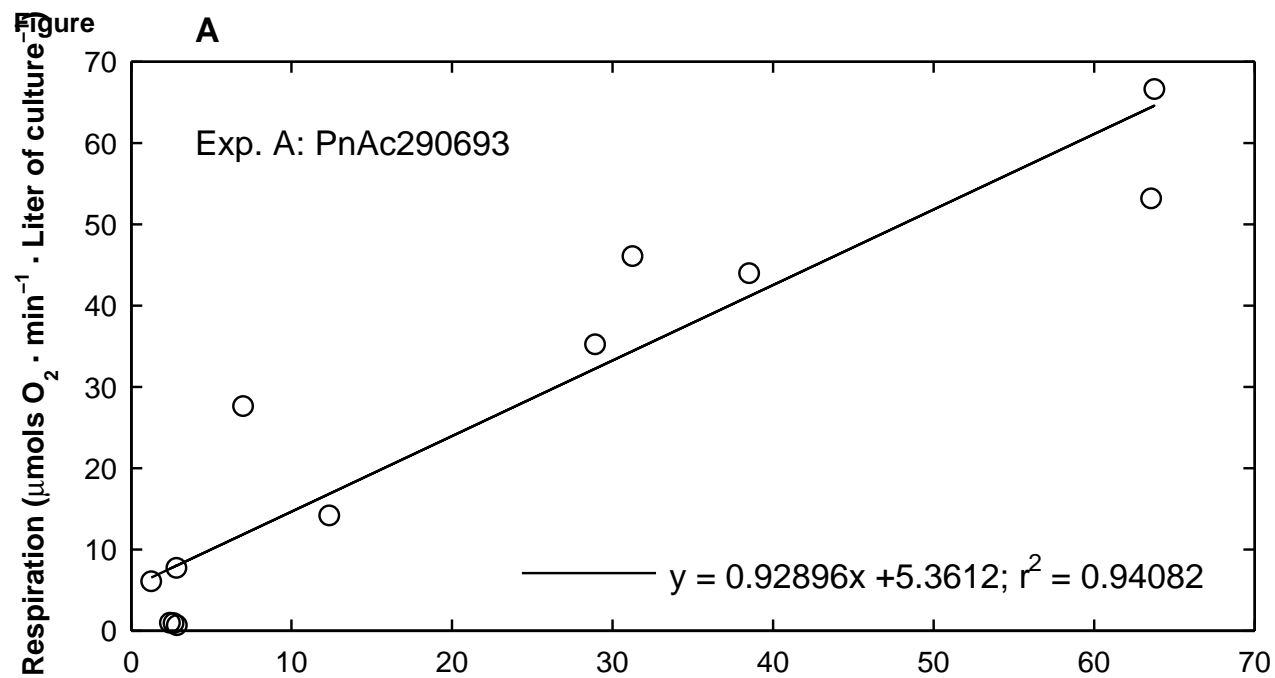
Figure



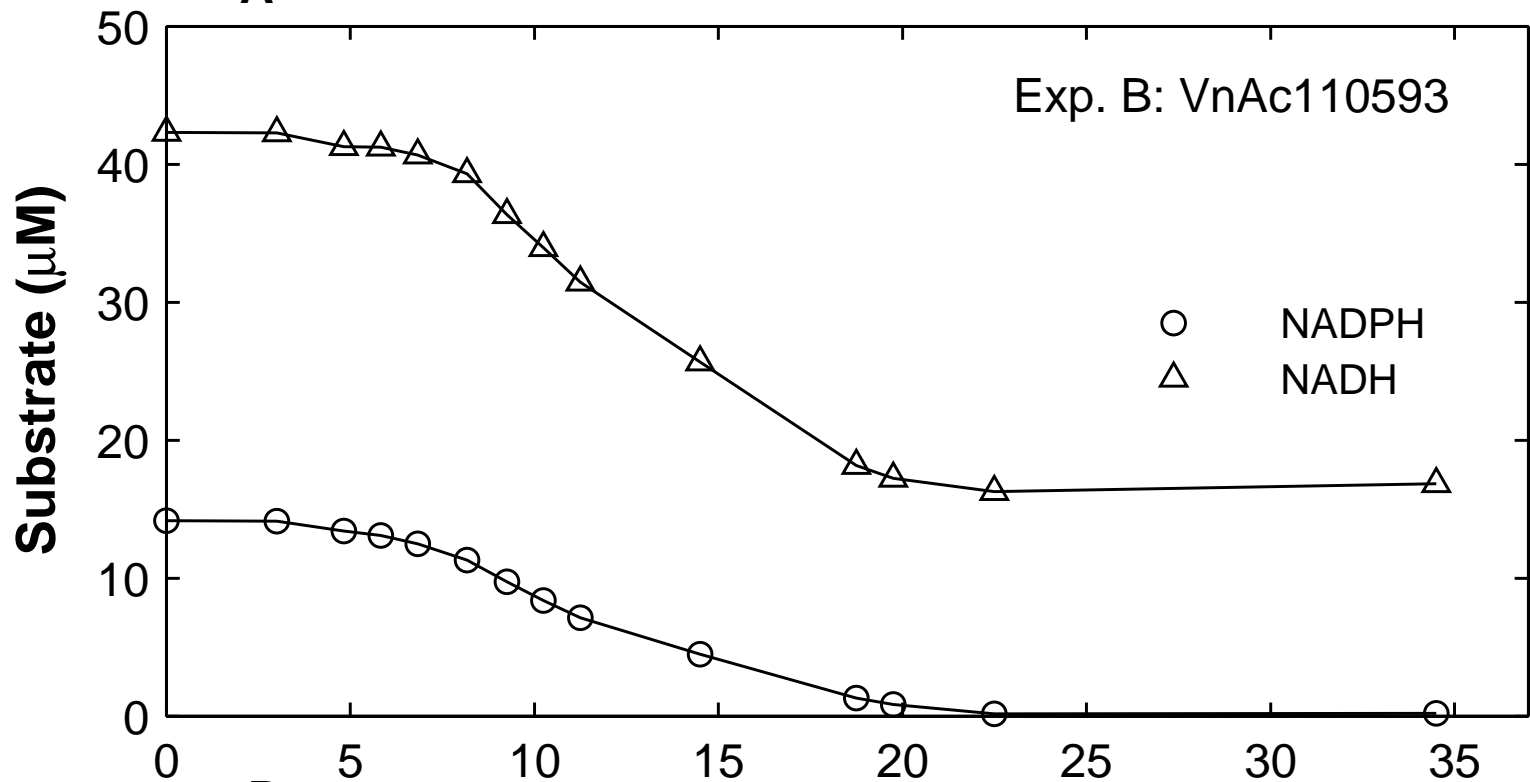
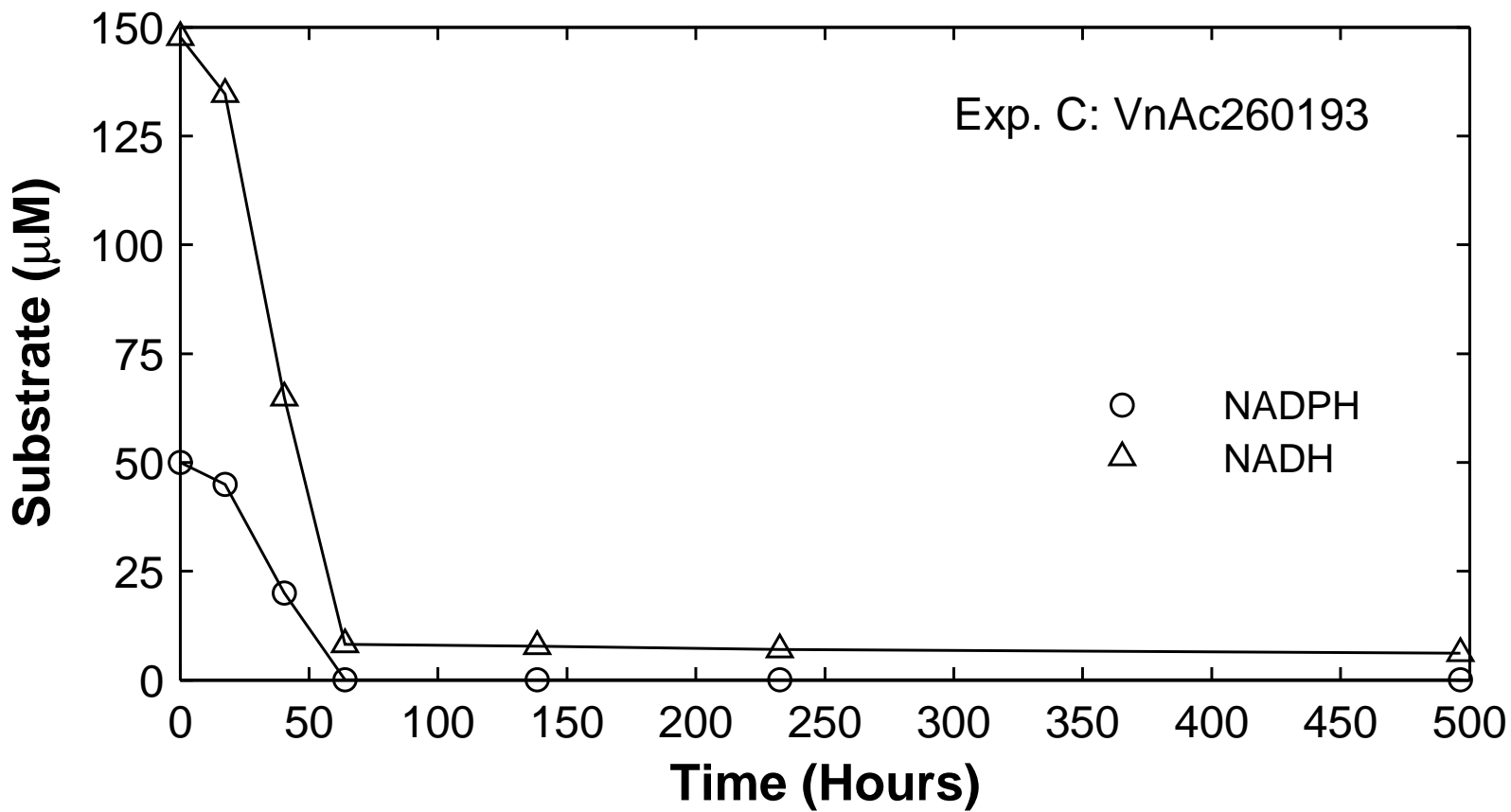
Figure

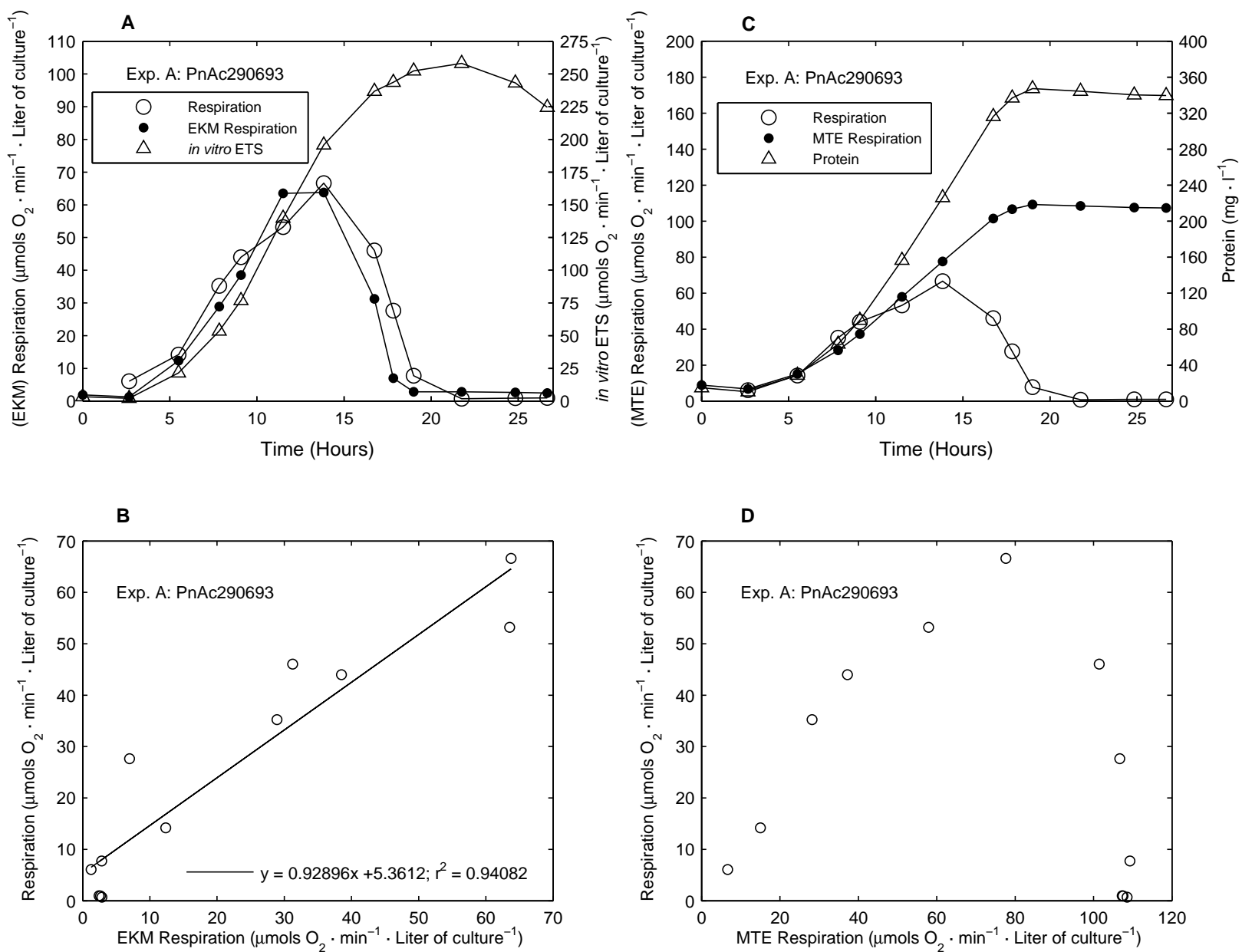




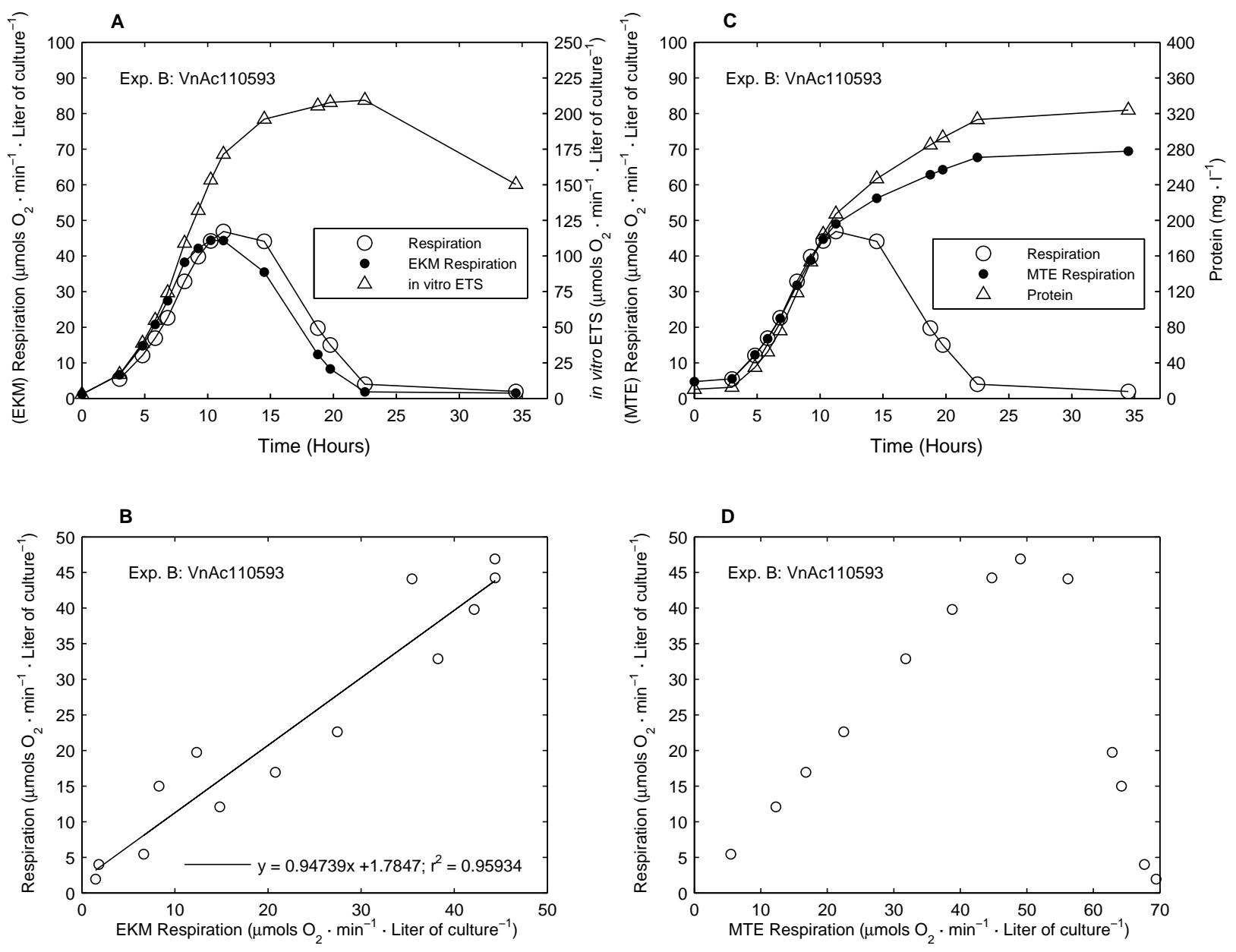


Figure

**A****B**

**Figure**



**Figure**

**Figure**