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SHORT COMMUNICATION

Batch production of coenzyme Q10 by recombinant *Escherichia coli* containing the decaprenyl diphosphate synthase gene from *Sphingomonas baekryungensis*

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Abstract Coenzyme Q_{10} (CoQ_{10}) is an important antioxidant used in medicine, dietary supplements, and cosmetic applications. In the present work, the production of CoQ_{10} using a recombinant *Escherichia coli* strain containing the decaprenyl diphosphate synthase from *Sphingomonas baekryungensis* was investigated, wherein the effects of culture medium, temperature, and agitation rate on the production process were assessed. It was found that Luria–Bertani (LB) medium was superior to M9 with glucose medium. Higher temperature (37 °C) and higher agitation rate (900 rpm) improved the specific CoQ_{10} content significantly in LB medium; on the contrary, the use of M9 medium with glucose showed similar values. Specifically, in LB medium, an increase from 300 to 900 rpm in the agitation rate resulted in increases of 55 and 197 % in the specific CoQ_{10} content and C_OQ_{10} productivity, respectively. Therefore, the results obtained in the present work are a valuable contribution for the optimization of CoQ_{10} production processes using recombinant *E. coli* strains.

Keywords Coenzyme Q · Oxygen supply · Agitation rate · Recombinant *Escherichia coli* · Culture medium

Introduction

Coenzyme Q (CoQ), also known as ubiquinone (UQ), is a lipid-soluble antioxidant that functions as electron transporter in the respiratory chain playing an important role in ATP biosynthesis [2, 10]. In addition, CoQ contributes to disulfide bond formation in bacteria proteins [12] and sulfide oxidation in yeast [24]. This molecule is formed from the conjugation of a benzoquinone ring and an isoprenoid chain with a characteristic length, depending on the organism. For example, human CoQ contains ten isoprenoid units (CoQ_{10}), *Saccharomyces cerevisiae* produces CoQ_6, and *Escherichia coli* produces CoQ_8 [13]. CoQ_{10}, the CoQ form present in humans, is also naturally produced by other organisms such as *Agrobacterium tumefaciens*, *Paracoccus dinitrificans*, *Rhodobacter sphaeroides,* and *Sphingomonas baekryungensis* [5, 6, 28].

CoQ_{10} has been used for the treatment and prevention of several human diseases such as Parkinson´s disease, Alzheimer´s disease, heart diseases, and diabetes [13]. CoQ_{10} deficiency has been associated with several clinical conditions, where oral CoQ_{10} supplementation has successfully reversed some of the symptoms in many patients [8]. In addition, CoQ_{10} has been used as a dietary supplement and, in cosmetics, for the formulation of anti-aging creams [11, 17]. CoQ_{10} applications are increasing rapidly; hence, new and more productive processes are thus required. Current approaches involve the use of natural producers increasing CoQ_{10} levels by the selection of mutants with elevated specific CoQ_{10} content and the optimization of fermentation parameters using these strains. However, the natural producers tend to be difficult to apply in industrial settings [7]. For instance, *A. tumefaciens* generates exopolysaccharides that produce viscosity problems in the culture medium and difficult product extraction [23]. Therefore, another important strategy has been to develop recombinant *E. coli* strains for CoQ_{10} biosynthesis [19]. As mentioned above, *E. coli* is not a native CoQ_{10} producer; however, production of CoQ_{10} could be achieved by

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replacing the native octaprenyl diphosphate synthase (IspB) by a decaprenyl diphosphate synthase (DPS) from a native CoQ_{10} producer [6, 20, 21, 27].

Several *E. coli* strains have been constructed for the production of CoQ_{10}, where the main focus has been the introduction of different genetic modifications, primarily by overexpressing genes related to CoQ biosynthetic pathway [6, 15, 25, 27]. Most *E. coli* CoQ_{10} production experiments have been performed using the complex Luria–Bertani (LB) medium or a defined medium using glucose as the sole carbon source [4, 15, 20, 27]. To the best of our knowledge, the effect of culture medium composition on CoQ_{10} production in recombinant *E. coli* strains has not yet been addressed.

On the other hand, very limited information is available about the effect of operating conditions on specific CoQ content in *E. coli*. Wild-type *E. coli* possesses three different quinones: CoQ_8, menaquinone 8 (MK_8), and demethylmenaquinone 8 (DMK_8). It has been reported that in aerobic conditions, CoQ_8 represents 65 % of the total isoprenoid quinones produced, whereas DMK_8 and MK_8 represent 32 and 3 %, respectively. In contrast, under anaerobic conditions CoQ_8, DMK_8 and MK_8 correspond to 7, 42, and 51 % of the total isoprenoid quinones, respectively [22]. Even though CoQ_8 biosynthesis is favored in aerobic conditions in wild-type *E. coli*, changes in oxygen supply would possibly affect specific CoQ_8 content. There is evidence that a limited oxygen supply favored CoQ_{10} synthesis in natural producer strains, *Agrobacterium* and *Rhodobacter* [3, 26]. In particular, Choi et al. [3] reported an increase in specific CoQ_{10} content with a decrease in air flow rate, a decrease in oxygen transfer rate, or a decrease in the agitation rate from 600 to 450 rpm in *Agrobacterium* cultures. On the other hand, Li et al. (2003) have reported the effect of different operating conditions (temperature, agitation rate, initial pH) in a complex medium on CoQ_{10} production in shake flasks using an *E. coli* BL21 (Δ*ispB::ddsA*) strain, where the decaprenyl synthase gene, *ddsA*, was cloned from *R. radiobacter* [16]. The authors reported that higher temperatures (36–39 °C) resulted in lower specific CoQ_{10} contents in complex medium stating that the DPS from *R. radiobacter* may be more unstable than other DPS from a different origin; therefore, the effect of culture temperature in a different strain may not be extrapolated from these results. In addition, the authors evaluated the effect of the agitation rate in CoQ_{10} production in shake flasks using a range of 0–240 rpm, showing that higher specific CoQ_{10} contents are achieved at higher agitation rates [16]. However, no evaluation of higher agitation rates was performed, or the effect of the agitation rate in different culture media. The present work reports bioreactor experiments performed at higher agitation rates (300–900 rpm) with both complex and defined media to evaluate the effect of the agitation rate in CoQ_{10} production in a recombinant *E. coli* expressing a DPS from *S. baekryungensis.*

Hence, the aim of the present work was to evaluate the effect of culture medium, temperature, and agitation rate on CoQ_{10} production in a recombinant *E. coli* strain as an important contribution to the development of better industrial processes for CoQ production.

Materials and methods

Strain and plasmid

The strain used in the present work was the engineered *E. coli* CC2.D strain, which is based in the wild-type *E. coli* MG1655 with a deletion in the endogenous octaprenyl diphosphate synthase, coded by the *ispB* gene, to avoid coenzyme Q_8 formation, and the expression of the plasmid pDPTSo containing the decaprenyl diphosphate synthase gene (dps_o) from *S. baekryungensis* to produce CoQ_{10} [6].

Culture medium and inoculum preparation

The effect of the culture medium on CoQ_{10} production was assessed using a defined medium and a complex medium. M9 medium ($Na_2HPO_4*7H_2O$ 12.8 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L, NH_4Cl 1 g/L, $MgSO_4$ 0.24 g/L, $CaCl_2$ 0.011 g/L, thiamine 0.001 g/L) supplemented with 4 g/L glucose was used as the defined medium, and Luria–Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) was used as the complex medium. Both media were supplemented with 100 mg/L ampicillin for plasmid conservation. Initial pH was adjusted to 7.2. The inoculum was prepared in 500-mL shake flasks with 100 mL of the corresponding culture medium according to the experiment. The cultures were incubated at 37 °C and 250 rpm in an orbital shaker overnight.

Shake flasks experiments

Shake-flask experiments were performed in 500-mL shake flasks with 100 mL of the corresponding culture medium at 30 or 37 °C and 250 rpm in an orbital shaker. 1 mM IPTG was added to induce dps_o expression from pTDPSo when 0.4–0.5 OD was reached. Culture samples' volumes corresponding to 20 mg of dry cell weight (DCW) were collected and centrifuged at 10000 rpm, for 10 min, and at 4 °C. The supernatant was used for glucose determination (in the defined medium experiments). Cell pellets from the end of the exponential phase were stored at −20 °C overnight before solvent extraction for CoQ_{10} determination. Each shake flask experiment was performed in triplicate.

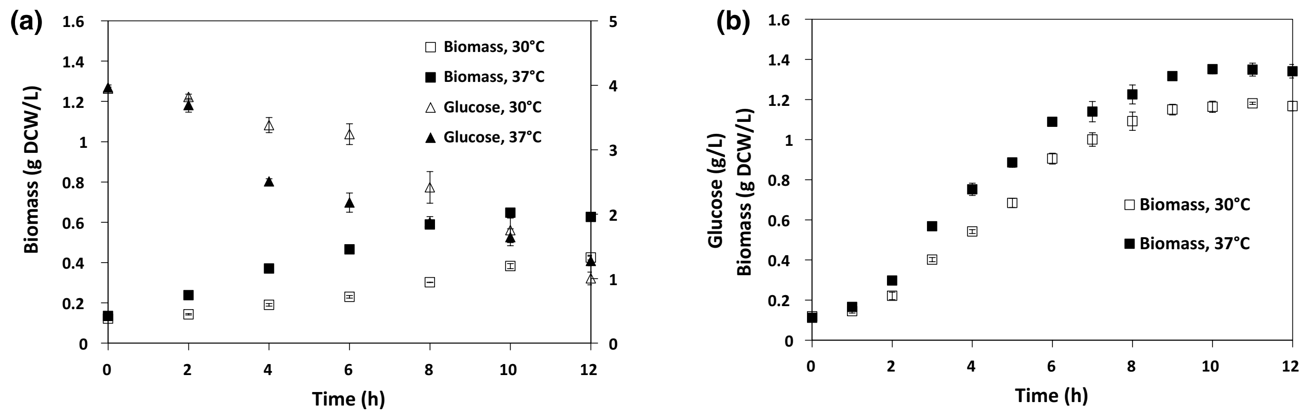

Fig. 1 *E. coli* CC2.D cultures in shake flasks with different culture media and temperature. Cell growth and glucose concentration profiles in M9 with glucose (**a**) and cell growth profile in LB (**b**) medium. *DCW* dry cell weight. Values represent mean ± SD of three replicates

Bioreactor experiments

Bioreactor experiments were performed in a 3-L Bioflo 115 (New Brunswick) bioreactor using 1.5 L of culture medium. The operating conditions were 37 °C; pH 7.2; agitation rates of 300, 600, and 900 pm; and air flow rate of 1.5 L/min. Each bioreactor experiment was performed in triplicate.

Biomass determination

Culture growth was followed by spectrophotometer measurements at 600 nm. Dry cell weight was calculated using a calibration curve.

Glucose analysis

Glucose concentration was determined using the dinitrosalicylic acid reagent method [18].

Coenzime Q_{10} analysis

Frozen pellets were resuspended in 6 mL of ice-cold methanol (Merck) followed by 6 mL of petroleum ether (boiling point 40–60 °C, Merck). The mixture was vortexed for 1 min and then centrifuged at 10000 rpm, for 5 min and at 4 °C. The upper petroleum ether phase was removed and transferred to a clean tube. Another 3 mL of petroleum ether was added to the lower phase. Then, the mixture was vortexed and centrifuged as described earlier, and the petroleum ether phases were combined and let to evaporate to dryness under a nitrogen flow. The dried extracts were stored at −20 °C. Then, dried extracts were resuspended in 500 µL ethanol HPLC grade (Merck), filtrated (22 mm), and 10 µL was injected into the HPLC system (Perkin Elmer, serie 200), using a Symmetry C18 column 5 µm 4.6 × 150 mm (Waters, Milford, MA, USA) at room temperature, UV detector 243 nm, and ethanol:methanol (70:30) as mobile phase at a flow rate of 1 mL/min. Calibration curves were prepared using CoQ_{10} standard (Sigma-Aldrich Co., St. Louis, MO, USA).

Results and discussion

Effect of culture medium and temperature in CoQ_{10} production

Shake flasks experiments were performed with defined medium (M9 with glucose) and complex medium (LB) at 30 and 37 °C. Cell growth and glucose profiles are presented in Fig. 1. CoQ_{10} production results are shown in Table 1. In general, LB medium was superior for CoQ_{10} production showing significantly higher biomass concentrations and CoQ_{10} productivity than M9 with glucose medium at the corresponding temperatures (Table 1). No significant difference in specific CoQ_{10} content was found in M9 with glucose at both temperatures. On the other hand, the specific CoQ_{10} content increased by 34 % when the temperature was increased from 30 to 37 °C in LB medium. This result is opposite to the results reported by Li et al. (2003) where higher temperatures showed lower specific CoQ_{10} content in an *E. coli* strain expressing a DPS from *R. radiobacter.* Li et al. [16] state that the DPS from *R. radiobacter* could be more unstable than others; therefore, the temperature response seems to be strain dependent and the results with one strain could not be extrapolated to another.

Overall, a fourfold CoQ_{10} productivity increase was achieved by increasing the culture temperature from 30 to 37 °C and changing from M9 with glucose medium to complex LB medium. Then, 37 °C was selected for the bioreactor experiments.

Table 1 Effect of culture medium and temperature in CoQ_{10} production in recombinant *E. coli* shake flasks cultures

Medium	Temperature (°C)	Biomass (g DCW/L)	Biomass yield (g DCW/g glucose)	Specific CoQ_{10} content (mg/g DCW)	CoQ_{10} productivity (mg/Lh)
M9 + Glucose	30	0.376 ± 0.013	0.119 ± 0.017	0.420 ± 0.045	0.016 ± 0.001
	37	0.659 ± 0.018	0.221 ± 0.008	0.430 ± 0.018	0.035 ± 0.001
LB	30	1.106 ± 0.032	N/A	0.420 ± 0.024	0.052 ± 0.003
	37	1.375 ± 0.011	N/A	0.564 ± 0.030	0.086 ± 0.005

Values represent mean ± SD of three replicates

DCW dry cell weight, *N/A* not applicable

Effect of the agitation rate on CoQ_{10} production

Agitation rates of 300, 600, and 900 rpm were evaluated for CoQ_{10} production in recombinant *E. coli* strain CC2.D. Figure 2 shows cell growth, glucose consumption, and specific CoQ_{10} profiles as well as the dissolved oxygen tension (DOT) profiles for bioreactor experiments. The results of CoQ_{10} production are presented in Table 2.

The maximum specific CoQ_{10} content in all cases was achieved at the end of the growth phase, and the values decreased at later times during the stationary phase. With the LB medium, maximum specific CoQ_{10} content increased in about 50 % when the agitation rate was increased from 300 to 600 rpm, a further increase in agitation rate, to 900 rpm, showed a twofold increase in CoQ_{10} productivity, compared with the result at 300 rpm, without increasing the maximum specific CoQ_{10} content significantly (Table 2). On the other hand, specific CoQ_{10} content was similar when M9 with glucose was used at all the different agitation rates (Table 2). Similarly, Li et al. (2003) reported an increase in specific CoQ_{10} content with an increase in agitation rate from 0 to 240 rpm in shake flasks using LB medium; however, no higher agitation rates were evaluated, and no experiments were performed with defined medium for comparison [16]. On the other hand, the agitation rate in the CoQ_{10} produced the opposite effect in recombinant *E. coli* strains compared to the results obtained with *Agrobacterium*, a natural CoQ_{10} producer, in a complex medium [3]—where a higher agitation rate resulted in lower specific COQ_{10} content and productivity. This is probably related to the fact that *E. coli* possesses three different isoprenoid quinones and adjusts their composition according to the environmental conditions, whereas *Agrobacterium* possesses only one type of quinone. Shestopalov et al. [22] have shown that native CoQ_8 biosynthesis in *E. coli* is favored in aerobic conditions over the other two isoprenoid quinones (MK, DMK), while the opposite was true in anaerobic conditions. However, the authors did not evaluate the effect of different oxygen supply levels on CoQ_8 production.

In the present work, the highest CoQ_{10} productivity was reached at the highest agitation rate evaluated, when the LB medium was used. This could indicate that a condition with a higher oxygen transfer rate would possibly increase it even further. In that sense, future studies could use higher agitation rates and/or higher flow rates to test this hypothesis.

Interestingly, the effect on CoQ_{10} production at different agitation rates reported in the present work was culture medium dependent, where no differences in specific CoQ_{10} content were observed when M9 with glucose medium was used (Table 2). This may be related to a possible glucose-inhibitory effect on the expression of CoQ biosynthetic genes in *E. coli* [9, 29]. In addition, the biosynthesis of CoQ requires several precursors, and the use of glucose as only carbon source may limit the amount of each precursor available for CoQ biosynthesis, whereas the use of a complex medium may favor the increased availability of precursors for CoQ production.

Chorismate is a precursor used not only for CoQ production but also for the synthesis of aromatic amino acids, such as phenylalanine. Similar to the present work, Khamduang et al. [14] have reported an increase in phenylalanine production with an increase in agitation rate from 200 to 400 rpm in bioreactor experiments. On the other hand, the isopentenyl diphosphate is a precursor of the isoprenoid chain in CoQ, as well as a precursor for heterologously produced terpenes, as lycopene. Alper et al. [1] have reported a significant increase in lycopene production in recombinant *E. coli* when the agitation rate was increased from 150 to 400 rpm. Therefore, the increase in agitation rate may be increasing the metabolic flow through the biosyntheses of both CoQ precursors, leading to a higher specific CoQ_{10} content in recombinant *E. coli* strains.

Zhang et al. [28] used BL21 (DE3) strain harboring different plasmids expressing the *dps* gene from *A. tumefaciens* either alone or with *ubiCA*, *ubiA*, or *ubiG*. The results those authors showed (Table 1, [28]), for a strain expressing *A. tumefaciens*, DPS are significantly lower than the results

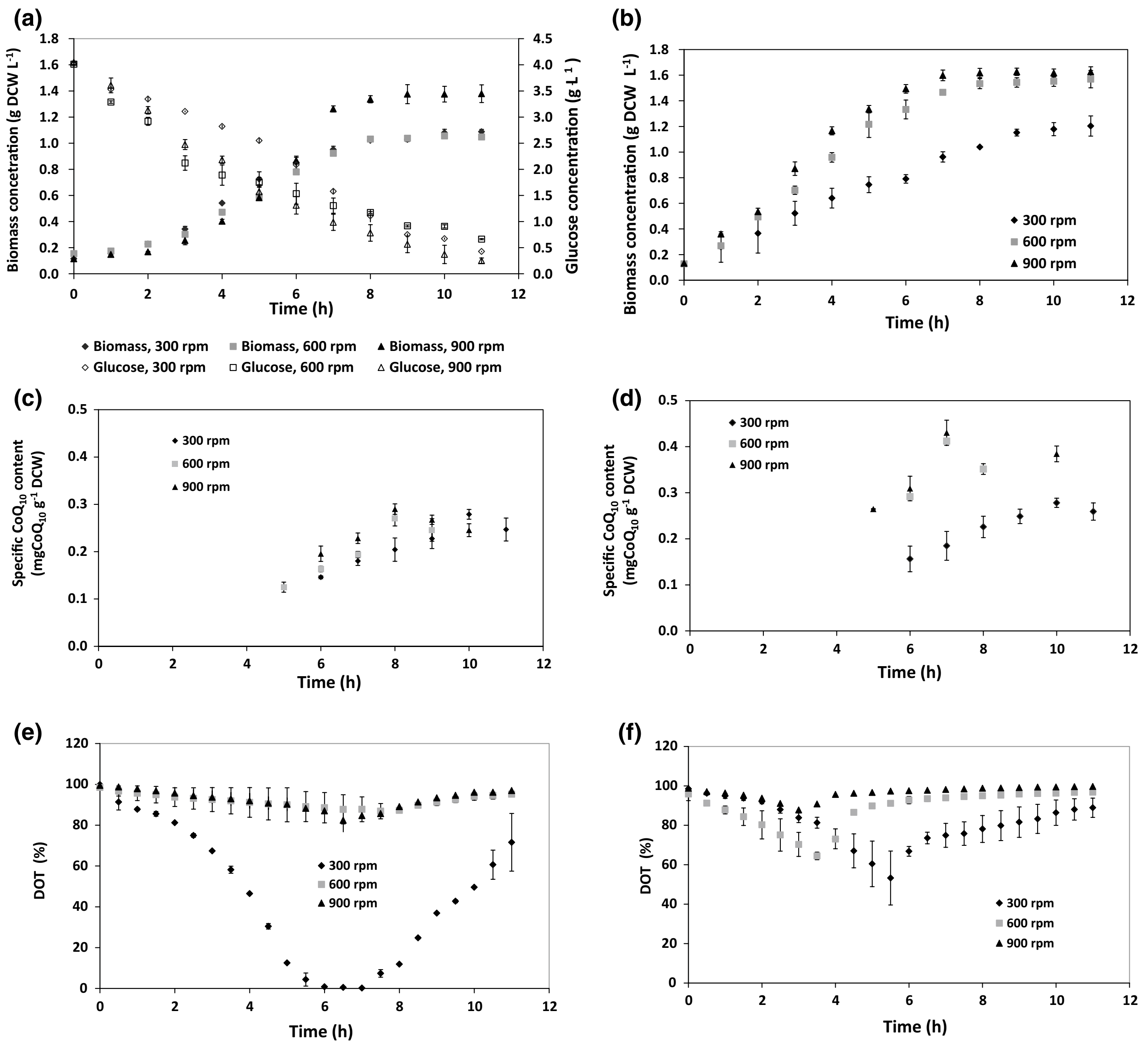

Fig. 2 *E. coli* CC2.D culture in bioreactor with different culture media and agitation rates. Cell growth and glucose concentration profiles in M9 with glucose (**a**) and LB (**b**) medium. Specific CoQ_{10} content profiles in M9 with glucose (**c**) and LB (**d**) medium. Dissolved oxygen tension (DOT) profiles in M9 with glucose (**e**) and LB (**f**) medium. *DCW* dry cell weight. Values represent mean ± SD of three replicates

reported by our group (0.15 vs. 0.56 mg CoQ_{10}/g DCW) for complex medium in shake flasks at 37 °C. Higher specific CoQ_{10} contents were reported by the same authors for other strain constructions which include the overexpression of *ubiCA* genes [28]. Other reports [4] show that the expression of DPS from different plasmids resulted in different specific CoQ_{10} contents; one of their strains, where the *dps* gene was expressed from a p15a origin plasmid, showed results similar to our experiments, and another strain where the *ddsA* gene was overexpressed from a constitutive plasmid showed higher specific CoQ_{10} content [4]. Therefore, the reported specific CoQ_{10} contents have shown to be dependent on the genetic modifications, *dps* source, expression vectors, operating conditions, and culture medium.

Conclusion

The effects of culture temperature and agitation rate were culture medium dependent. Increases in agitation rate and temperature resulted in increases of biomass concentration, specific CoQ_{10} content, and productivity in LB medium;

Table 2 Effect of culture medium and agitation rate on CoQ_{10} production in recombinant *E. coli* batch bioreactor cultures

Medium	Agitation rate (rpm)	Biomass (g/L)	Biomass yield (g DCW/g glucose)	Maximum specific CoQ_{10} Content (mg/g DCW)	Maximum CoQ_{10} productivity[a] (mg/L/h)
M9 + Glucose	300	1.088 ± 0.018	0.271 ± 0.003	0.279 ± 0.010	0.030 ± 0.001
	600	1.047 ± 0.001	0.267 ± 0.001	0.270 ± 0.016	0.035 ± 0.000
	900	1.378 ± 0.068	0.333 ± 0.022	0.290 ± 0.011	0.048 ± 0.001
LB	300	1.203 ± 0.079	N/A	0.278 ± 0.010	0.033 ± 0.001
	600	1.569 ± 0.068	N/A	0.412 ± 0.008	0.086 ± 0.001
	900	1.626 ± 0.039	N/A	0.430 ± 0.017	0.098 ± 0.003

Values represent mean ± SD of three replicates

DCW dry cell weight, *N/A* not applicable

[a] Maximum productivity was reached at the same time as maximum specific CoQ_{10} content in all cases

however, in M9 with glucose medium, the effect of the agitation rate was significant for biomass concentration and CoQ_{10} productivity, but no differences in maximum specific CoQ_{10} content were observed. These results are crucial information that could be applied for the development of more efficient CoQ_{10} production processes using recombinant *E. coli* strains.

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