Development of a Cost-effective Medium for Photorhabdus Temperata Bioinsecticide Production From Wastewater and Exploration of Performance Kinetic

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Research

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Development of a cost-effective medium for *Photorhabdus temperata* bioinsecticide production from wastewater and exploration of performance kinetic

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Abstract

This study investigates the optimization of culture conditions for enhancing *Photorhabdus temperata* biopesticide production using wastewater (WS4) as a raw material. Box-Behnken design (BBD) was used to evaluate the effect of carbon to nitrogen ratio (C/N), NaCl concentration and inoculum size on *Photorhabdus temperata* biomass production and insecticidal activity. Modelling results suggest that the selected variables had contributed significantly to the responses. For enhanced biopesticide production the optimum operation conditions were as follow: inoculum size=4 %; C/N ratio=12.5 and sodium chloride concentration= 4 g/L for two responses. 1.95 and 2.75 folds improvement in oral toxicity and biomass production, respectively, were obtained when using the three variables at their optimum values. From batch fermentations carried out in the cost-effective medium developed in this study (WS4 I) and WS4 used as control, *P. temperata* kinetic parameters in term of biomass production and substrate consumption were modeled. The obtained results showed that the maximum specific growth rate was of 0.38 h^{-1} compared to 0.16 h^{-1} obtained in WS4. In addition, the efficiency of *P. temperata* to metabolize organic carbon was enhanced by optimizing culture conditions reaching 70 % instead of 47.2 % in the control fermentation. Under the optimized conditions, *P. temperata* cells showed higher specific consumption rate resulting in *P. temperata* toxin synthesis improvement.

Key words: *Photorhabdus temperata;* industrial wastewater; response surface methodology; bioinsecticide production; kinetic parameters.

Nomenclature

μ_{max}	maximum specific growth rate (h^{-1});
q_S	specific substrate consumption rate (g / [g_x h]);
r_S	substrate consumption rate (g / [L h]);
r_X	biomass production rate (g_x / [L h]);
S	substrate concentration (mg/ L);
X	biomass concentration (g_x / L);
t	time (h);
d_t	doubling time (h).

Introduction

Microbial biopesticide based on *Bacillus thuringiensis* are vastly well-known as being a control agent for target pest. However, insects evolving resistance to *B. thuringiensis* delta-endotoxins have been emerged and caused consequently a profound threat to subsequent use of these toxins in insect control programs (Xiao and Wu 2019). *Photorhabdus luminescens* is a promising candidate for biological control. This entomopathogenic bacterium kills insects through the secretion of a range of toxins including Toxin complexes (Tcs) (Meusch et al. 2014), *Photorhabdus* insect related (Pir) toxins (Waterfield et al. 2005), Makes caterpillars floppy (Mcf) toxins (Ullah I et al. 2014) and *Photorhabdus* Virulence Cassettes (PVC) (Yang et al. 2006). Additionally, *Photorhabdus* produces secondary metabolites which are effective as protein toxins (Stock et al. 2017). Through these varieties of toxins, this insect pathogenic bacterium is able to infect a broad range of insect hosts belonging to the order of Lepidoptera including *Helicoverpa armigera*, *Spodoptera litura* and *S. exigua*. Indeed, injection of purified Txp40, derived from *Photorhabdus akhurstii*, reduced number, viability of insect hemocytes after 12 h incubation and increased significantly the phenoloxidase activity of insect hemolymph leading to melanization reaction and larval death (Shankhu et al. 2020). Moreover, *Plutella xylostella* larvae mortality was observed after oral administration of *P. luminescens*. Mortality rates of *P. xylostella* were of 18.89 % and 91.11 %, after administration of fermentation broth or supernatant, respectively (Wu et al. 2020). *Ephestia kuehniella* is another target insect belonging to the order of Lepidotera. 100 % mortality of this insect larvae was reached after oral administration of broth medium at a concentration of 12×10^8 cells/mL (Jallouli et al. 2013). Moreover, mortality of protonymph, deutonymh, adult males and adult females of the polyphagous pest *Tetranychus urticae*, belonging to the order of Trombidiformes was recorded after oral administration of *P. temperata* cell free supernatant (Eroglu et al. 2019). Diptera is another insect order susceptible to *Photorhabdus*

toxins. Indeed, *P. luminescens* suspension had a significant oral toxicity on *Drosophila suzukii* larvae and pupae, with mortalities up to 70-100 % after 10 days of treatment (Shawer et al. 2018). In addition, larvae of *Aedes aegypti* and *Ae. albopictus* belonging to this same order were demonstrated to be orally susceptible to *P. luminescens* subsp. *akhurstii* with a mortality of 98 % after 96 h of treatment (Yooyangket et al. 2018).

Although considerable success has been made through the use of *Photorhabdus* as bioinsecticide against different insect order at a lab scale, improvement of low-cost *P. temperata* biopesticide production remains a challenge for industrial production of this bacterium. In this context, Tunisian industrial wastewater (WS4) has been evaluated for potential application of low-cost feedstock. However, in this medium, low *P. temperata* biomass production and oral toxicity, against the Mediterranean flour moth *E. kuehniella* were obtained, which were of 4×10^8 cells/mL and 42 %, respectively (Keskes et al. 2020). Improvement of *P. temperata* biomass production was well studied in two media: the optimized medium (OM) based on glucose and yeast extract (Jallouli et al. 2008) and the complex medium (CM) based on soya bean meal (Jallouli et al. 2011), but it was never reported in wastewater. Enhancement of *P. temperata* cells production is achieved essentially by adding sodium chloride at 5 g/l. Indeed, at such concentration NaCl doubled biomass production, increased culturability and biological activity in both studied media (Jallouli et al. 2011). Moreover, maintaining glucose at 4 g/l in the OM increased significantly *P. temperata* biomass production ((Jallouli et al. 2012).

Based on the literature survey, it has been found that C/N ratio and inoculum size influenced greatly fermentation process in wastes and wastewaters. Indeed, adjustment of C/N at 45 during facultative co-digestion of palm oil mill effluent and empty fruit bunch was demonstrated to enhance methane production (Nurliyana et al. 2015). Varying C/N ratio between 7.9 and 9.9 by using combination of sludge increased *B. thuringiensis*

entomotoxicity, growth rate and viable cell count (Vidyarthi et al. 2002). Inoculum was identified also to affect fermentation in Agaricus bisporus wastewater to produce *Saccharomyces cerevisiae* and in distillery wastewater for hydrogen gaz production (Huang et al. 2019; Wicher et al. 2013). Moreover, optimization of inoculum volume during *B. thuringiensis* biopesticide production in waste activated sludge resulted in higher spores, specific growth rate and entomotoxicity value (Lachhab et al. 2001).

Thus, the objective of this study is to identify the optimal conditions for improving the biomass production and insecticidal activity of *P. temperata* grown in industrial wastewater by using Box-Behnken design (BBD). To the best of our knowledge, there are no report on enhancement of biomass or/and oral toxicity of *P. temperata* by using response surface methodology (RSM). Here, NaCl concentration, C/N ratio and inoculum size were taken as three factors of BBD and biomass production and insecticidal activity were considered as the responses of the system. *P. temperata* growth kinetics in the newly optimized medium and WS4 were also evaluated improving our knowledge about *P. temperata* growth behavior and total organic carbon (TOC) consumption efficiency using mathematical models.

Material and methods

Microorganisms

P. temperata ssp. temperata strain K122 and *P. luminescens* strain Q 167/2 were used in the present work. The K122 strain was used for bioinsecticide production because of its high toxicity to the Lepidopterean insect larvae *E. kuehniella*. *P. luminescens* strain Q 167/2 is a non pathogenic bacterium, used as a negative control in the bioassay (Jallouli et al. 2013).

Biopesticide production media

In this study, three media were used: Luria-Bertani (LB) medium, wastewater (WS4) demonstrated to be a suitable medium for *P. temperata* biopesticide production (Keskes et al.

2020) and the newly optimized medium (WS4 I). WS4 was sampled from the food industry STL (Société Tunisienne de Levure, Beja, Tunisia) and its composition is presented in Table 1. All the media were sterilized at 121°C for 15 min and the pH was adjusted to 7.0 ±0.1.

Inocula preparation and growth experiments

One 48 h old colony of *P. temperata* strain K122 was isolated and dispersed into 3 ml of LB medium and incubated overnight at 30 °C. Inoculum was used to inoculate 500 ml Erlenmeyer flasks containing 85 ml of WS4, with initial optical density of 0.025 at 725 nm (Jallouli et al. 2008). In order to study the effect of inoculum type, a second pre-culture was prepared by inoculating 250 ml Erlenmeyer flask containing 50 ml of WS4 with 1 mL from the first one preculture for 10 h of incubation at 30°C and an agitation of 200 rpm. In this case, different volumes corresponding to different inoculum sizes (1, 2, 3, 4 and 5 %) were used to inoculate 500 ml Erlenmeyer flasks. Incubation was carried at the optimized conditions for biopesticides production (Jallouli et al. 2008).

C/N ratio

As shown in Table 1, WS4 has a C/N ratio of 4.53. As glucose was demonstrated to be an easily assimilated carbon source by *P. temperata* cells (Jallouli et al. 2008), it was selected to adjust the C/N ratio in WS4, avoiding the difference in the availability of the carbon source provided by the use of another effluent or waste containing high carbon concentration. In the presented work, glucose was added from a stock solution (20 %) to obtain a specific C/N ratio varying between 4.53 and 30. This ratio was calculated based on the carbon present in both WS4 and glucose and nitrogen content in WS4.

Experimental design and optimization by response surface methodology

To improve *P. temperata* strain K122 biopesticide production in wastewater, an experimental design was developed by RSM. A three-level BBD was used to explore the effects of three

independent variables which are: C/N ratio (X_1), inoculum size (X_2) and sodium chloride concentration (X_3) (Table 2). Biomass production and insecticidal activity presented as the total cell count (Y_1) and growth inhibition of *E. kuehniella* larvae (Y_2) were considered as response parameters. The optimization step required 12 experiments and six replicates for the center point which are performed in order to check the validity of the fitted model (Table 3). Each experiment was done in triplicate and an average value of the response was used for the presentation of the results. The obtained data from BBD were subjected to analysis of variance (ANOVA) to check for errors and the significance of each parameter (Table 4). Then, data were subjected to a multiple regression analysis to obtain a second-order polynomial regression equation fitted for *P. temperata* biopesticide production (equation 1).

$$Y= \beta_0+\sum_{i=1} \beta_i x_i + \sum_{i=1} \beta_{ii} x_i^2+\sum_{i=1} \sum_{j=i+1} \beta_{ij} x_i x_j \quad \text{(Eq. 1)}$$

Where, Y is the predicted response; xi and xj are independent coded variables; β_0 is a interception coefficient; β_i, β_{ii} are linear and quadratic regression coefficients, respectively; β_{ij} are regression coefficients of interaction between two variables. Regression analysis, analysis of variance (ANOVA) and response surface plots of the experimental data were performed by using the statistical software NEMROD (Mathieu et al. 2000).

To select the effective range of the experimental variables (Table 2), preliminary experiments were conducted with a broad concentration range of NaCl (0.5-10 g/L) and C/N (4.53-30) which were individually supplemented to WS4. Our findings shrink these ranges to (2-6 g/L) and (5-20) as showing in Table 2. Besides, by using two different inocula grown respectively, in LB and WS4, the same growth rate and the same biomass production were obtained. Interestingly, a rapid entry in the exponential phase was achieved by using WS4 as a medium for inoculum preparation (data not shown). Consequently, the use of WS4-grown inoculum as a second step in inoculum preparation was adopted in this study. Likewise, by keeping all

other variables at fixed concentrations and varying the inoculum size (0.5-9 %), the range between 1 and 7 % was selected to design the experimental run.

Analytical procedures

Samples collected after 48 h of fermentation were subjected to determination of total cell count (Jallouli et al. 2008) and insecticidal activity (Jallouli et al. 2013). Total direct count was determined microscopically using Thoma counting chamber at 100-fold magnification. Insecticidal activity against the flour moth *E. kuehniella* was assessed as the growth inhibition of the fed larvae with K122, compared to growth of similar larvae number fed with the non-toxic *P. luminescens* strain Q by using similar cell count of 4×10^8 cells/mL.

For kinetic study, *P. temperata* biomass production and substrate concentration during fermentation in WS4 and WS4 I were determined. *P. temperata* cell concentration was determined gravimetrically. Samples were periodically taken from the fermentation broth, centrifuged (13 000 rpm for 5 min), washed twice with saline water (9 %) and dried at 105 °C in pre-weighted porcelain vials until constant weight. The total solids (TS) content of WS4 before inoculation was subtracted from all TS samples to obtain TS equivalent to biomass production at each incubation time. The ability of *P. temperata* cells to metabolize the organic carbon for its growth was monitored during fermentation in WS4 and WS4 I by determination of TOC concentration carried out with Shimadzu TOC analyzer TOC-VCPH at each incubation time according to standard methods (APHA, 1992).

Mathematical modeling

As growth substrates (carbohydrate and nitrogen) present in wastewater were considered to be in excess during the batch fermentations, the exponential growth rate could be expressed as first order. Thus, *P. temperata* kinetic parameters (r_x, r_s, μ and q_s) could be determined from mathematical models illustrated in equations from (2) to (5). In this study, production rate of

K122 toxins was not estimated because until now there is no method allowing toxin quantification.

$$\frac{dX}{dt} = rx = \mu X \quad (\text{Eq. } 2)$$

$$\frac{dS}{dt} = -rs \quad (\text{Eq. } 3)$$

$$\frac{rx}{X} = \mu \quad (\text{Eq. } 4)$$

$$\frac{rs}{X} = qs \quad (\text{Eq. } 5)$$

A logistic model (LIS EXCEL) in microsoft Excel software was used to adjust the obtained profiles and to calculate *P. temperata* kinetic parameters.

Statistical analysis

All results related to determination of TS and TOC were the average of three replicates of three separate experiments. They were statistically analyzed by SAS software (Version 6) using Duncan test performed after analysis of variance (ANOVA).

Results and discussion

Response surface methodology: Box-Behnken Design

In this study, we tried to analyze model and interpret the experimental data using RSM as a mathematical modeling system. In this regard, a twelve-run BBD design with 3 levels and 3 factors with six replications at the central point was designed to study the optimum combination of NaCl concentration, C/N ratio and inoculum size for maximum biomass production and insecticidal activity of the insect pathogenic bacterium *P. temperata*. The experimental design along with experimental results is presented in Table 3.

The analysis of variance (ANOVA) of the response surface quadratic model for biomass production and growth inhibition of *E. kuehniella* larvae was presented in Table 4. The

obtained results showed that Fischers's *F*-test and *p*-values reveal significance for both regression models. Moreover, according to Table 4 the lack of fit is not significant for both responses. Consequently, both models could predict the optimal biomass production and insecticidal activity and define optimal variable values. As shown in Table 5, the coefficients of determination (R^2) were of 0.989 and 0.991, for biomass and oral toxicity responses, respectively, indicating that 98.9 % and 99.1 % of the variability in the response could be explained by the model which reflects a good correlation between experimental and predicted values. The adjusted coefficient of determination values (Adj R^2 =0.976 and 0.981, respectively) were within reasonable agreement with predicted R^2.

The second order polynomial regression equation fitted into the experimental data for total cell count response (Y_1) is as follows (Equation 6):

$$Y_1 = 10.5 + 0.16\ X_1 + 2.38\ X_2 + 1.59\ X_3 - 0.43\ (X_1^2) - 3.125\ (X_2^2) - 1.69\ (X_3^2) + 0.30\ (X_1 X_2) - 0.39\ (X_1 X_3) + 0.31\ (X_2 X_3) \quad \text{(Eq. 6)}$$

The significance of each coefficient, determined by *p*-values, is summarized in Table 5. The *p*-values imply that the first and second order main effects of X_2 and X_3 are significant. However, none of the interaction effects are significant. Moreover, the fitted equation for prediction of *P. temperata* cell toxicity (Y_2) is as follows (Equation 7):

$$Y_2 = 80.28 + 5.81\ X_1 + 16.80\ X_2 + 7.21\ X_3 - 9.78\ (X_1^2) - 16.49\ (X_2^2) - 22.25\ (X_3^2) + 7.02\ (X_1 X_2) + 0.68\ (X_1 X_3) + 1.30\ (X_2 X_3) \quad \text{(Eq. 7)}$$

As shown in Table 5, the first and second order main effects of X_2 and X_3 and the second order of X_1 are found to be significant, as well as the interaction effect between X_1 and X_2.

Surface and contour plots generated by using the software NEMROD are presented in Fig. 1 and Fig. 2. These figures were plotted to examine the relationship between the different paired factors and to determine the optimum of each one for the highest biomass production and insecticidal activity. As shown in Fig. 1a, biomass production increases with increasing

231 inoculum size and sodium chloride concentration to reach a maximum value of 11.4×10^8
232 cell/mL obtained at range of 4-6.5 % and 3.8-6 g/L, respectively. Similarly, previous studies
233 reported that addition of sodium chloride at 5 g/l to OM and CM doubles biomass production
234 of the strain K122 of *P. temperata*. NaCl was demonstrated to be a stimulator of growth of the
235 strain K122 by increasing nutrients assimilation (Jallouli et al. 2011). In addition, Jallouli et
236 al. (2012) demonstrated that biomass production increased with the increase in inoculum size.
237 Indeed, by increasing inoculum size from 0.05 to 0.15 optical density unit, biomass increased
238 both in LB medium and OM. Moreover, Lachhab et al. (2001) showed improvement of *B.*
239 *thuringiensis* total viable cell count and spore count by varying inoculum size from 2 to 4 %.
240 However, increasing inoculum size of *P. temperata* above 6.5 %, at optimal NaCl
241 concentration of 4 g/L, has a negative effect on biomass production (Fig. 1a). Inhibition of *P.*
242 *temperata* cell growth could be explained by the fact that high initial K122 cell concentration
243 resulted in rapid consumption of oxygen and nutrients resulting in low final biomass
244 production. By plotting NaCl concentration or inoculum size against C/N ratio (Fig. 1 b and
245 Fig 1 c), the obtained results showed that C/N ratio seems to have no significant effect on
246 biomass production when inoculum size and NaCl concentration were at low levels. In
247 contrast, when WS4 was supplemented by an inoculum volume and sodium chloride
248 concentration upper than 4.5 % and 4 g/L, respectively high biomass production was obtained
249 (11.4×10^8 cell/mL). This high level was reached only when adjusting the C/N ratio to a range
250 of 9-15 (Fig. 1 b) and a range of 9.8-20 (Fig. 1c). Thus, it is evident that keeping a balanced
251 composition of C/N in wastewater, by adding available carbon source, is required for
252 improving the total cell production. Similarly, Wisuthiphaet and Napathorn (2016) reported
253 that using an optimal C/N ratio when culturing *Azohydromonas lata* on various cane sugar
254 products improved its growth rate and its productivity. Additionally, Wang et al. (2007)

255 reported that optimization of C/N ratio of agricultural food wastes, enhanced polymer
256 productivity.
257 To illustrate the interaction effect between inoculum size, NaCl concentration and C/N ratio
258 for maximum insecticidal activity, contour plots were plotted (Fig. 2). The obtained results
259 showed that maximum toxin synthesis occurred when increasing inoculum size and sodium
260 chloride concentration beyond 4 % and 3.5 g/L, respectively (Fig. 2 a). The enhancement of
261 *P. temperata* toxin synthesis through NaCl addition was demonstrated by Jallouli et al.
262 (2011). Indeed, an improvement of 81.4 and 42.22 % of *P. temperata* oral toxicity in CM and
263 OM, respectively was obtained when NaCl was added beyond 2 g/l. Moreover, this study
264 demonstrates for the first time the involvement of inoculum size and C/N ratio in K122 toxin
265 synthesis. Indeed, according to Fig. 2b, these variables have a positive effect on *P. temperata*
266 toxicity. The highest toxicity of 85 % was obtained by using inoculum volume and C/N of 5.5
267 % and 12.5, respectively. At optimal C/N value of 12.5, exceeding inoculum size range
268 between 4.3 and 7 %, reduced considerably *P. temperata* toxicity. This fact could be due to
269 low biomass production obtained by using high and low initial cell concentration resulting in
270 decrease of the final entomotoxicity. Similar findings were reported by Lachhab et al. (2001),
271 when studying *B. thuringiensis* entomotoxicity in wastewater sludge. It is particularly
272 important to note that the lowest toxicity of 15.3 % was obtained at (-1) and (+1) levels of
273 C/N along with (-1) and (+1) levels of NaCl concentration (Fig. 2c). This could be explained
274 by the fact that at high and low levels there is a decline in *P. temperata* structural metabolism
275 affecting toxin gene expression. These results were in agreement with those reported by
276 Vidyarthi et al. (2002) demonstrating that it is necessary to optimize the C/N ratio during *B.*
277 *thuringiensis* fermentation in sludge to enhance its entomotoxicity. Therefore, from the
278 optimization plots, the maximum response of biomass production and insecticidal activity
279 occurred at an inoculum size of 4 %, NaCl at 4 g/L and a C/N ratio of 12.5. At these

conditions total cell count and oral toxicity were of 11.4×10^8 cell/mL and 85 % which correspond to an improvement of 185 and 102.38 %, respectively compared to WS4 which is interesting from a practical point of view in the production of low-cost *P. temperata* bioinsecticide. To experimentally validate the predicted response, *P. temperata* fermentations were carried out by using the newly optimized medium WS4 I and WS4 inoculated by overnight preculture carried out in LB medium at initial optical density of 0.025. The validation experiment carried out under optimized conditions showed that the experimentally determined biomass production value (11×10^8 cell/mL) and oral toxicity (82 %) were in agreement with the statistically predicted one (11.4×10^8 cell/mL and 85 %), confirming the model's authenticity.

Determination of specific rates of cell growth and substrate consumption

To achieve a modeling for large scale *P. temperata* biopesticide production in industrial wastewater, kinetic models that relate consumption of total organic carbon and biomass production during batch fermentation are required. This is the first report in literature focusing on the mathematical modeling of *P. temperata* growing in wastewater as a low-cost feedstock. Fig. 3 shows growth curves and substrate consumption in optimized medium WS4 I and WS4 considered as control. From the obtained results, it is clear that *P. temperata* growth was enhanced by optimizing the growth conditions (NaCl concentration, inoculum volume and C/N ratio). Indeed, biomass concentration in WS4 increased slightly ($p < 0.05$) from 0.56 g/L obtained at 4 h incubation to 1.3 g/L after 10 h incubation. However, in the RSM optimized medium biomass production started to increase from 2 h reaching a high level of 2.91 g/L after the same incubation time. Thus, using the optimized conditions for *P. temperata* biopesticide production in batch fermentation reduced the lag phase by 2 h and increased the final biomass production. In addition, determination of the maximum specific growth rate μ_{max} from the slope of the semi-logarithmic plot of biomass production in WS4 versus

fermentation time showed a value of 0.16 h^{-1} (R^2: 0.99) ($p < 0.001$). Interestingly, grown in WS4 I as a fermentation medium, *P. temperata* cells, reached the exponential phase with a higher μ_{max} of 0.38 h^{-1} (R^2: 0.99) ($p < 0.001$), which is interesting from a practical point of view for the production of *P. temperata* bioinsecticide. Enhancement of maximum specific growth rate indicates improvement of *P. temperata* cells metabolism by optimizing growth conditions. The obtained value of μ_{max} is consistent with previous report on *P. luminescens* indicating a maximum specific growth rate varying between 0.36 h^{-1} and 0.33 h^{-1} in medium based on nutrient broth and complex nutrients, respectively (Belur et al. 2013).

In addition results of Fig. 3, analyzed according to Duncan test performed after ANOVA, showed that total organic carbon concentration decreased significantly from 1.8 g/L and 2.2 g/L at inoculation to 0.95 g/L and 0.66 g/L after 10 h of fermentation in WS4 and WS4 I, respectively. This indicates a TOC consumption efficiency of 47.2 % and 70 %, respectively after 10 h of incubation. The slight increase in initial TOC level in the optimized medium is due to glucose addition for adjusting the C/N ratio at 12.5. Increase in TOC consumption efficiency could be confirmed from results illustrated in Fig. 4. Indeed, by plotting TOC consumption versus fermentation time almost linear curves were obtained with a slope of 0.09 and 0.17 in control fermentation and the RSM optimized medium, respectively indicating improvement in the ability of the strain K122 to metabolize organic carbon available in wastewater. Enhancement of nutrient assimilation by the strain K122 could be explained by sodium chloride addition at 4 g/L in WS4 I as demonstrated for CM and OM used for *P. temperata* bioinsecticide production (Jallouli et al. 2011).

Comparison between specific growth rate and specific consumption rate of substrate for WS4 and WS4 I is presented in Fig. 5. The close association between *P. temperata* cell growth and substrate consumption can be observed from the variations of the specific growth rate (μ) and specific substrate consumption rate (qs) in these two fermentation processes. In general,

increase in specific growth rate was followed by decrease in specific consumption rate and vice versa. As shown in Fig. 5, the variation in specific growth rate in WS4 and WS4 I followed similar patterns with a high value of 0.17 h^{-1} at 4 h incubation in WS4 and 0.29 h^{-1} at 2 h incubation in WS4 I corresponding to 4.07 h and 2.4 h of doubling time (d_t). The obtained specific growth rates differs from previously reported (μ = 0.36 h^{-1}, d_t = 2.1 h) obtained for *P. luminescens* in nutrient broth (Singh et al. 2012) and those reported by Orozco-Hidalgo et al. (2019) (0.21 h^{-1}, d_t=3.3 h) obtained by culturing *P. luminescens* subsp. *akhurstii* SL0708 in medium based on 10 g/L yeast extract and 3 g/L glucose. Likewise, O'Campo et al. (2017) reported that by using Yoo medium for *P. luminescens* growth, a specific growth rate of 1.4 h^{-1} was obtained corresponding to doubling time of 0.51 h. These variations could be attributed to bacterial isolates used and also for different media composition and culture conditions. Thus, it is evident that under the optimized conditions growth rate increased significantly with Pr > F value less than 0.001, throughout the fermentation giving higher *P. temperata* biomass production in reduced fermentation time. This finding contributes not only to the reduction of energy consumption during fermentation process but also to the enhancement of oral toxicity. *P. temperata* toxicity was demonstrated to be linearly related to biomass production during fermentation process in OM and wastewater used as an alternative medium (Jallouli et al. 2013; Keskes et al. 2020).

The Fisher's F test with a low probability value [(Pr > F value) <0.001] indicated that the specific consumption rate of substrate was enhanced in WS4 I with a maximum value of 0.36 (g / [g_x h]) compared to 0.14 obtained in WS4 (Fig. 5), confirming results obtained in Fig. 4. However, after 5 h incubation in WS4 I, qs gradually decreased to reach a value of 0.15 g / [g_x h] which is similar to that obtained in WS4. Improvement in substrate consumption rate during growth of K122 cells in wastewater may improve the specific metabolite production rate which is illustrated through the determination of insecticidal activity reaching 2.75 fold

compared to fermentation carried out under unoptimized conditions. The obtained results are in agreement with those reported by (Sarkar and Shimizu 2008) showing enhancement of lactate production rate by increasing substrate consumption rate.

Conclusion

The present research indicates that RSM was effective to optimize a combination of factors influencing *P. temperata* bioinsecticide production. The designed medium was useful for producing higher biomass production and insecticidal activity. Maximum toxicity and biomass production of 82 % and 11×10^8 cell/mL were obtained using the developed cost-effective medium. Under optimized conditions the specific growth and substrate consumption rates were improved leading to improvement of *P. temperata* toxin synthesis. Thus, enhancing biopesticide production in wastewater is a promising strategy to achieve low-cost and high active toxin production for large scale applications.

Abbreviations

P. temperata: Photorhabdus temperata; (WS4): Indusrial wastewater; (WS4 I): the newly optimized medium; CM: complex medium; OM: optimized medium; Tcs: Toxin complexes; Pir toxins: *Photorhabdus* insect related toxins; Mcf toxins: Makes caterpillars floppy toxins; PVC: *Photorhabdus* Virulence Cassettes; *S. exigua: Spodoptera exigua; P. xylostella: Plutella xylostella*:; *E. kuehniella*: *Ephestia kuehniella; Ae. Albopictus: Aedes albopictus;* BBD: Box-Behnken design; RSM: response surface methodology; TOC: total organic carbon; TS: total solids.

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Authors' contribution

KS performed fermentation and kinetic experiments, *JW* wrote the paper and conceived the experiments, *BAI* performed RSM design, *CM* designed and collected wastewaters characterization, *TS* developed the manuscript. All authors read and approved the final manuscript.

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389 Bioprocessing.

390 Competing interests

391 The authors declare that they have no competing interests.

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Table legends

Table 1: Characteristic of WS4

Table 2: Codes and values of independent variables at different levels

Table 3: Box-Behnken design matrix and results

Table 4: Analysis of variance

Table 5: Estimated regression coefficients corresponding to the Box-Behnken design

Figure legends

Figure 1: Contour plots of biomass production showing interactive effect of (a) inoculum size and NaCl, (b) NaCl and C/N, (c) inoculum size and C/N. the third parameter is at his central value.

Figure 2: Contour plots of insecticidal activity showing interactive effect of (a) inoculum size and NaCl, (b) inoculum size and C/N, (c) NaCl and C/N. the third parameter is at his central value.

Figure 3: Biomass production and TOC profiles during *P. temperata* fermentation in WS4 and WS4 I.

Figure 4: TOC consumption during *P. temperata* fermentation in WS4 and WS4 I.

Figure 5: Specific growth rate (μ) and specific substrate consumption rate (q_s) during *P. temperata* fermentation in WS4 and WS4 I

Table 1 :

Characteristics	WS4
Total solids (TS) (g/L)	7.1±0.11
Volatile solids (VS) (g/L)	4.3±0.22
Suspended solids (SS) (g/L)	0.6±0.05
Volatile suspended solids (VSS) (g/L)	0.6±0.04
pH	7.01
TOC (mg/L)	1828
Total nitrogen (mg/L)	403
C/N ratio	4.53

496 **Table 2 :**

Factor	Symbol	Level		
		-1	0	+1
C/N ratio	X1	5	12,5	20
Inoculum size (%)	X2	1	4	7
NaCl (g/L)	X3	2	4	6

497 **Table 3:**

Run	Coded levels			Results	
	C/N ratio X_1	Inoclum size (%) X_2	NaCl (g/L) X_3	Total cell counts (10^8 cells/mL)	Insecticidal activity (%)
1	-1	-1	0	5.00	40.40
2	1	-1	0	4.00	37.84
3	-1	1	0	9.30	56.13
4	1	1	0	9.50	81.66
5	-1	0	-1	6.00	37.40
6	1	0	-1	7.80	47.79
7	-1	0	1	9.75	47.34
8	1	0	1	10.00	60.44
9	0	-1	-1	2.00	15.34
10	0	1	-1	6.00	50.18
11	0	-1	1	4.75	30.30
12	0	1	1	10.00	70.34
13	0	0	0	10.50	80.16
14	0	0	0	10.75	83.79
15	0	0	0	10.75	80.80
16	0	0	0	10.50	77.90
17	0	0	0	10.50	78.87
18	0	0	0	10.00	80.14

498 **Table 4:**

Source	Total cell count(10^8cells/mL)					Insecticidal activity (%)				
	SS	df	MS	F	P	SS	df	MS	F	P
Model	130.43	9	14.49	76.88	<0.01***	7.64	9	8.49	98.07	<0.01***
Residual	1.50	8	0.18			6.93	8	8.66		
Lack of fit	1.13	3	0.37	5.03	5.8	4.90	3	1.63	4.03	8.4
Pure error	0.37	5	0.07			2.02	5	4.05		
Total	131.94	17				7.71	17			

*** Significant at the level 99.9%

Table 5:

Name	Total cell count (10^8cells/mL)				Insecticidal activity (%)			
	Coefficient	Error	t.exp	Significant	Coefficient	Error	t.exp	Significant
b 0	10.500	0.177	59.24	<0.01***	80.28	1.20	66.80	<0.01***
b 1	0.156	0.154	1.02	34.0	5.81	1.04	5.58	0.06
b 2	2.381	0.154	15.51	<0.01***	16.80	1.04	16.15	<0.01***
b 3	1.587	0.154	10.34	<0.01***	7.21	1.04	6.93	0.01***
b 11	-0.425	0.208	-2.04	7.3	-9.78	1.41	-6.94	0.01***
b 22	-3.125	0.208	-15.03	<0.01***	-16.49	1.41	-11.70	<0.01***
b 33	-1.688	0.208	-8.12	<0.01***	-22.25	1.41	-15.79	<0.01***
b 12	0.300	0.217	1.38	20.3	7.02	1.47	4.77	0.15**
b 13	-0.388	0.217	-1.78	11.0	0.68	1.47	0.46	66.0
b 23	0.313	0.217	1.44	18.6	1.30	1.47	0.88	40.6

R^2 (Y_1) = 0.989; Adj R^2 (Y_1) = 0.976; R^2 (Y_2) = 0.991; Adj R^2 (Y_2) = 0.981.
******* Significant at the level 99.9%
** Significant at the level 99 %

Figures

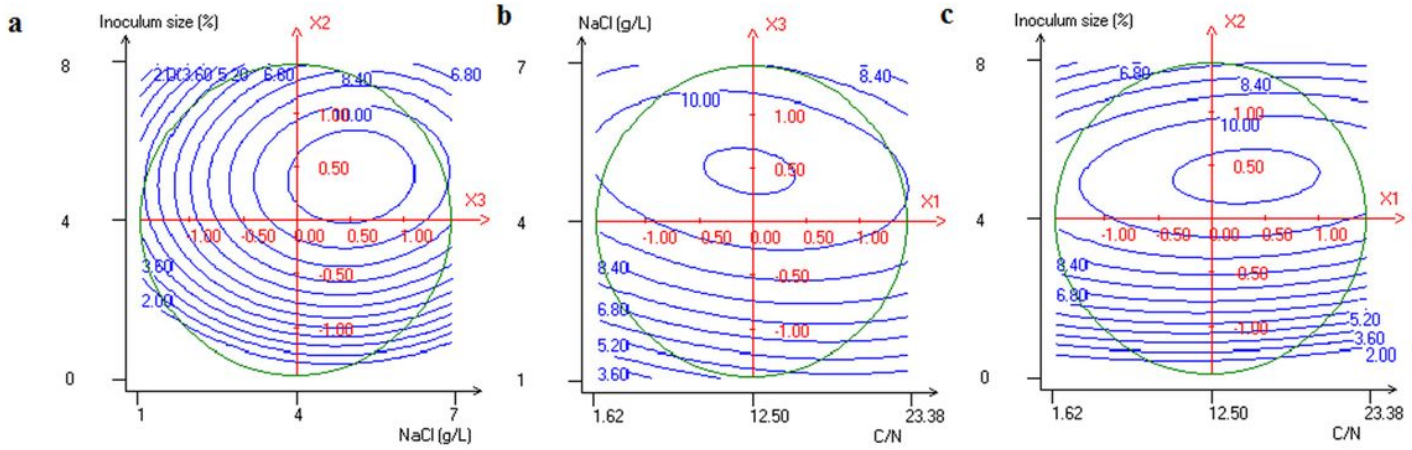

Figure 1

Contour plots of biomass production showing interactive effect of (a) inoculum size and NaCl, (b) NaCl and C/N, (c) inoculum size and C/N. the third parameter is at his central value.

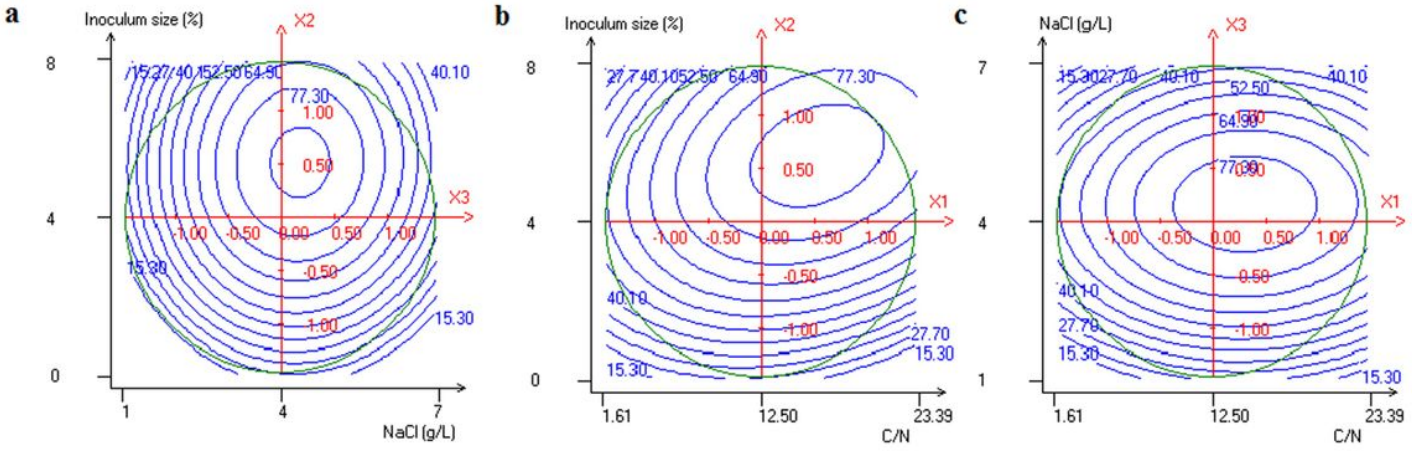

Figure 2

Contour plots of insecticidal activity showing interactive effect of (a) inoculum size and NaCl, (b) inoculum size and C/N, (c) NaCl and C/N. the third parameter is at his central value.

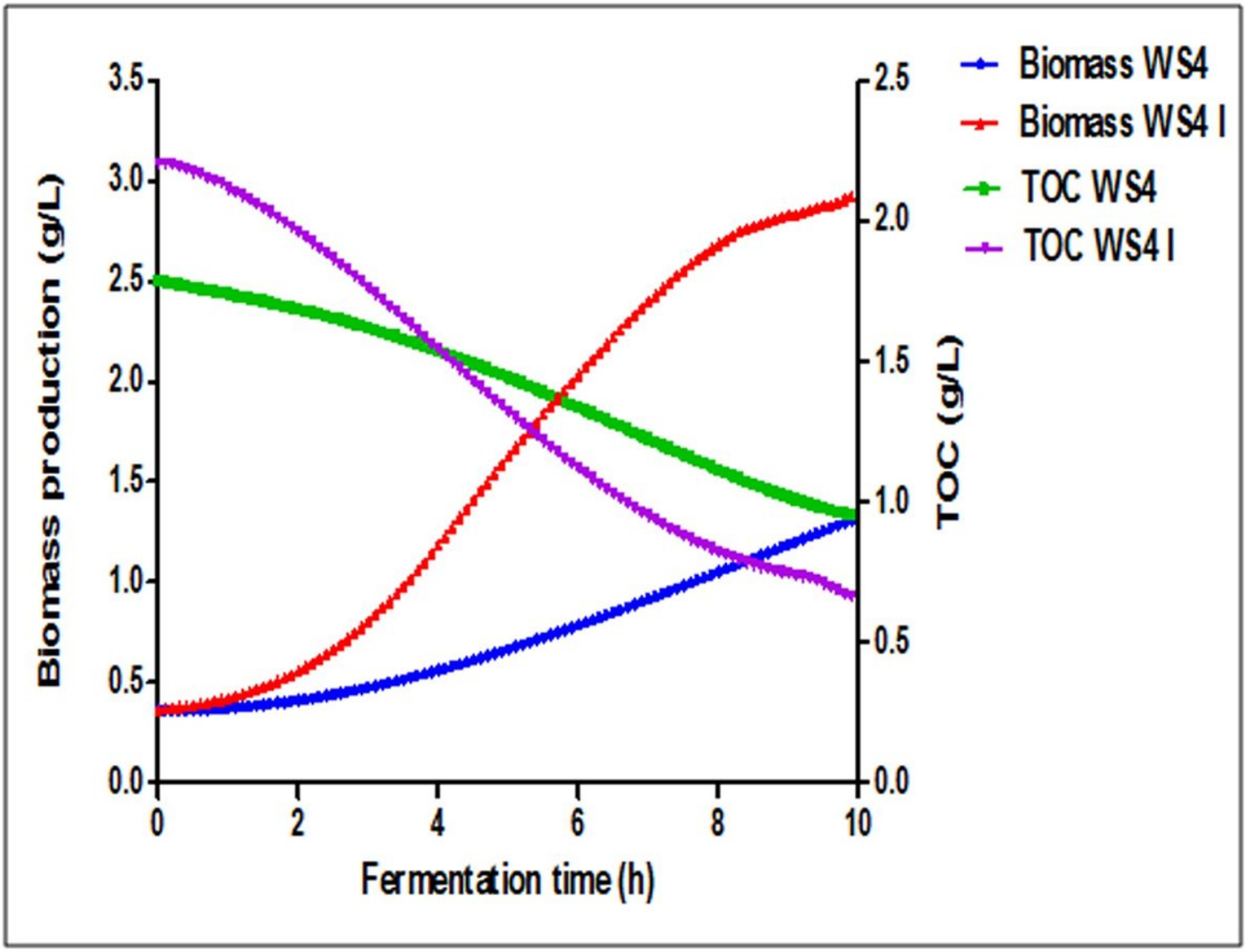

Figure 3

Biomass production and TOC profiles during P. temperata fermentation in WS4 and WS4 I.

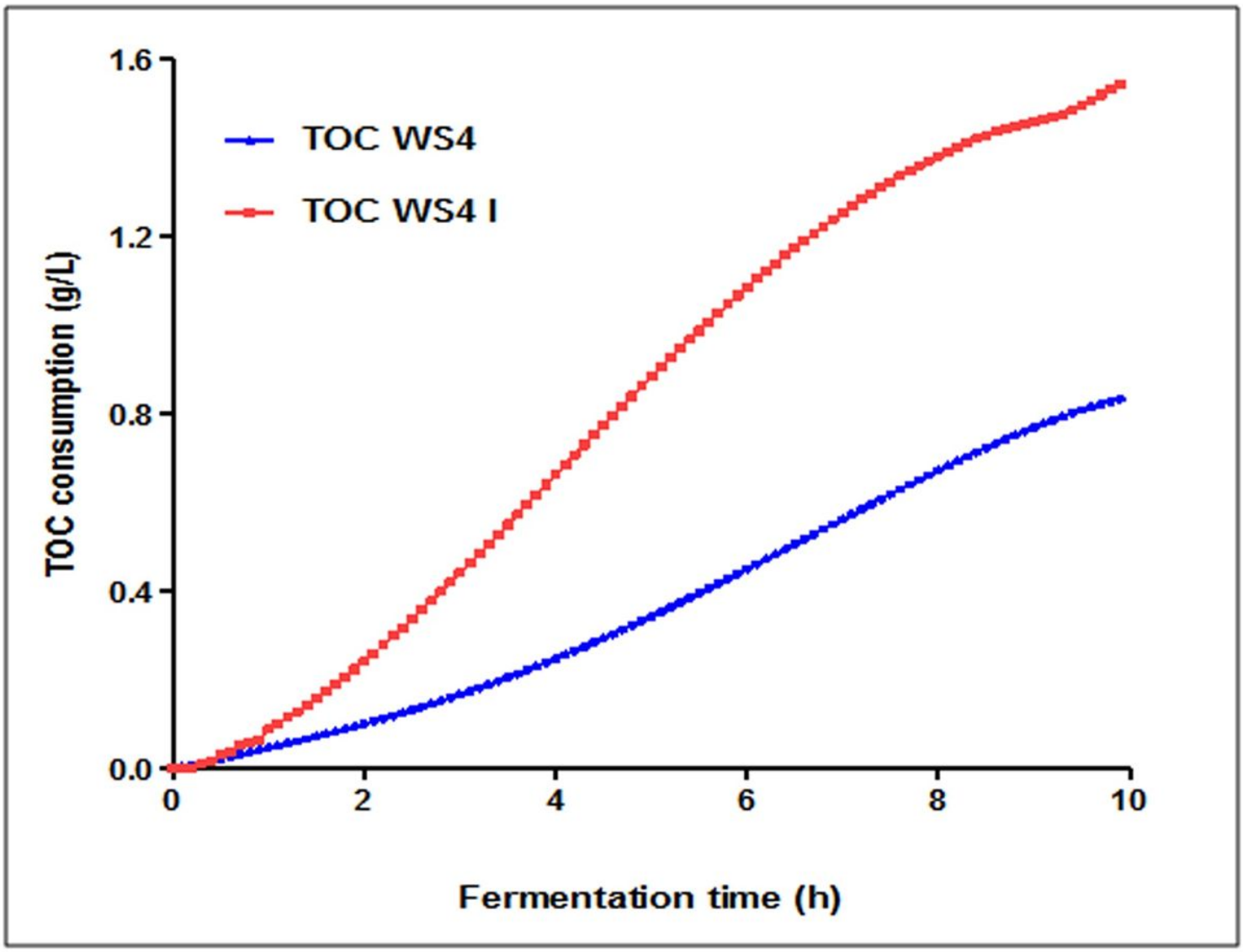

Figure 4

TOC consumption during P. temperata fermentation in WS4 and WS4 I.

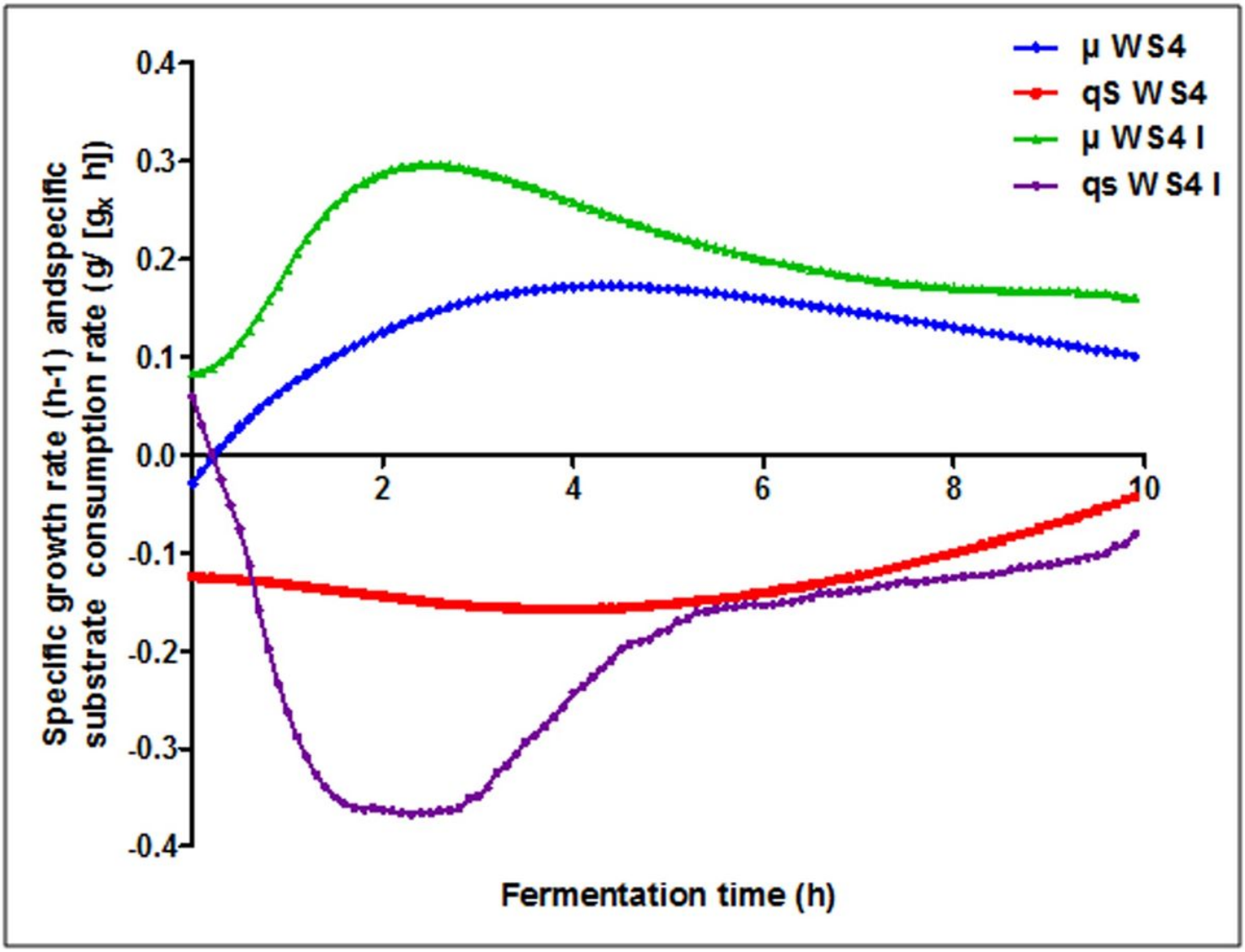

Figure 5

Specific growth rate (μ) and specific substrate consumption rate (qs) during P. temperata fermentation in WS4 and WS4 I

Supplementary Files

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- GA.pptx