Optimisation of culture composition for glyphosate degradation by *Burkholderia vietnamiensis* strain AQ5-12

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Abstract

The herbicide glyphosate is often used to control weeds in agricultural lands. However, despite its ability to effectively kill weeds at low cost, health problems are still reported due to its toxicity level. The removal of glyphosate from the environment is usually done by microbiological process since chemical process of degradation is ineffective due to the presence of highly stable bonds. Therefore, finding glyphosate-degrading microorganisms in the soil of interest is crucial to remediate this glyphosate. *Burkholderia vietnamiensis* strain AQ5-12 was found to have glyphosate-degrading ability. Optimisation of biodegradation condition was carried out utilising one factor at a time (OFAT) and response surface methodology (RSM). Five parameters including carbon and nitrogen source, pH, temperature and glyphosate concentration were optimised. Based on OFAT result, glyphosate degradation was observed to be optimum at fructose concentration of 6, 0.5 g/L ammonia sulphate, pH 6.5, temperature of 32 °C and glyphosate concentration at 100 ppm. Meanwhile, RSM resulted in a better degradation with 92.32% of 100 ppm glyphosate compared to OFAT. The bacterium was seen to tolerate up to 500 ppm glyphosate while increasing concentration results in reduced degradation and bacterial growth rate.

Keywords Glyphosate · *Burkholderia vietnamiensis* · One-factor-at-time (OFAT) · Response surface methodology (RSM) · Bioremediation

Introduction

N-Phosphonomethylglycine (glyphosate) has emerged as the most widely used herbicide in agriculture, silviculture and urban environments as the active ingredient in weed-killer formulations such as Roundup and WeedKiller™ (Borggaard and Gimsing 2008). It can be used in no-till agriculture to prepare the field prior to planting, during crop development and after crop harvest. This is because of glyphosate's unique ability to inhibit 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which is the key enzyme of shikimate pathway for aromatic compound biosynthesis in plant and several microorganisms. EPSP inhibition

suppresses the synthesis of proteins and secondary metabolites as well as de-regulates energy metabolism (Duke and Powles 2008).

The expansion of glyphosate-based herbicides was encouraged by the emergence of glyphosate resistant transgenic varieties of most significant agriculture species (soy, colza, maize, wheat, sugar beet and cotton), which accommodates 90% of all transgenic plant culture worldwide (Padgett et al. 1996). However, extensive use of glyphosate has resulted in its accumulation in soil and water environments. Moreover, adverse effects of glyphosate on animal metabolism have been reported (Sviridov et al. 2014). The main product of natural glyphosate degradation, aminomethyl phosphonic acid (AMPA), impairs the process of DNA repair and mRNA synthesis in both plants and animals (Richard et al. 2005). Glyphosate-contaminated soils exhibit a higher resistance to its toxicity and differ significantly in composition among the communities of normal soils (Dick and Quinn 1995). Glyphosate biodegradation by original or introduced microorganisms can be an effective means for removing glyphosate from soil and water.

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Environmental concerns toward glyphosate have compelled us to focus on the biodegradation of this herbicide. Although suitable microbial population may be accessible for biodegradation of a given contaminant, improvement of conditions is vital for the viability of the bioremediation process. A time effective and statistically designed response surface methodology (RSM) has been proposed for enhancement of different conditions for the remediation of environmental toxins (Ibrahim et al. 2015; Nawawi et al. 2016). RSM, supported by the software, is a pragmatic modelisation method used for estimating the relationship between a set of controlled experimental variables and the observed results. The application of optimisation in biotechnological system allows a more efficient use of resource while achieving enviable results (Ahmad et al. 2013; Suhaila et al. 2013; Yusuf et al. 2016).

The central composite design (CCD) with two-level full factorial design can create significantly better models compared to the other models (Karamba et al. 2016). This is because, CCD is very efficient in providing information on experiment variable effects and overall experimental error in a minimum number of required runs (El-Gendy et al. 2013). The use of this method has been successfully applied in optimization of medium composition (Ibrahim et al. 2005; Walia et al. 2015; Kumari et al. 2016). Nevertheless, fewer studies based on central composite design method are found in the literature regarding optimisation of degradation rate for glyphosate (Teófilo et al. 2004). Therefore, choosing CCD can provide crucial and new data on the biodegradation of glyphosate.

In this study, locally isolated glyphosate-degrading bacterium *B. vietnamiensis* strain AQ5-12 was subjected to optimisation using one factor at a time (OFAT) and response surface methodology (RSM) for comparing and optimising of medium constituents for glyphosate degradation. A central composite design matrix was used in optimisation experiments and iterative regression analysis performed to obtain optimal conditions for further applications in the biotechnological field. No reports from the literature are available regarding the optimization of biodegradation of glyphosate using the *Burkholderia* genus, and this work provides the avenue to study this bacterium. This provided a unique opportunity to further study this bacterium.

Materials and methods

Chemicals and culture conditions

Glyphosate PESTANAL@ 99.5% analytical standard was purchased from Sigma, Aldrich USA. Meanwhile, other chemicals used in media were obtained from Fisher (Malaysia). *Burkholderia vietnamiensis* strain AQ5-12 was

previously isolated from a glyphosate-contaminated site in Kedah, Malaysia (Manogaran et al. 2017). The bacterium was cultured in mineral salt media (MSM) containing g/L Tris buffer (1), glucose (5), NaCl (0.5), KCl (0.5) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), NH_4SO_4 (0.5), CaCl_2 (0.01) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.001) with pH range of 7.0 ± 0.2 . The media was sterilised at 121 °C in 15 psi for 15 min using Hirayama autoclave machine. A 50 ppm of glyphosate was added into the medium after the MSM cooled down to room temperature. Resting cells strain AQ5-12 were inoculated into 100 mL MSM consisting 50 ppm glyphosate. The culture media was incubated in an orbital shaker at 150 rpm in dark condition for 72 h.

Glyphosate assay

Each sample containing glyphosate was first oxidised into organophosphate according to the method of Glass (1981). The pH of each 1 mL sample was adjusted to 5.0 followed by adding 30% hydrogen peroxide to the samples. All samples were boiled at a moderate rate to dry. Samples were allowed to cool down at room temperature. To each 20–30 mL of sample, 0.25 M of HCl was added to aid in resolving the residue. Each 5 mL hydrogen peroxide treated sample was transferred into a test tube followed by the addition of 1 mL ascorbic reagent. The mixture was thoroughly mixed and allowed to stand for 10 min at room temperature. The absorbance of each solution in a 1.0 cm³ quartz cell was determined at 865 nm against blank. As a safe precaution, each piece of the glassware was thoroughly cleaned by washing with phosphate-free detergent followed by rinsing with dH₂O and soaked for 24 h in 0.5 M hot HCl, which was then rinsed using dH₂O to completely remove any phosphate residue. All the glasswares were tested using inorganic phosphate assay to ensure no phosphate residue contamination prior to running colorimetric assay using glyphosate.

Resting cells

Burkholderia vietnamiensis strain AQ5-12 was cultured in mass quantity in a sterilised broth medium comprising 10 g/L nutrient broth and 2 g/L yeast extract to accelerate microbial growth rate (Karamba et al. 2015). The strain was inoculated in 5 L conical flask containing broth medium. Aeration was introduced via a BB-800 aquarium air pump fitted with chrome tech MCE 0.45 µm syringe filter followed by incubation at room temperature for 48 h. Bubbles formed in the media was utilised as agitation. The bacterial cells were harvested at early stationary phase by centrifuging at 10,000×g with a temperature of 4 °C for 10 min. The harvested cells were washed twice using 10 mM Tris buffer and re-suspended in the same 10 mM Tris-base buffer solution. The optical density at 600 nm was adjusted to the range of

0.9–1.0 and stored at 8 °C for further use. This was done to standardize the inoculum's size.

Optimisation of degradation and growth condition via one-factor-at-time by free cells

Optimisation of *B. vietnamiensis* strain AQ5-12 was conducted to obtain the maximal growth and degradation activity of the bacteria. Several parameters such as pH, temperature, nitrogen source, carbon source and glyphosate concentration were used to study growth and degradation rate. Culture medium with the following compositions in 1 L of dH₂O was used in this study: glucose (5 g), NaCl (0.5 g), KCl (0.5 g), NH₄SO₄ (2 g), MgSO₄·7H₂O (0.2 g), CaCl₂ (0.01 g) and FeSO₄·7H₂O (0.001 g) (Benslama and Boulahrouf 2013). Meanwhile, the carbon sources used in the experiment are D-arabinose, D-mannitol, fructose, glycerol, glucose, maltitol, lactose starch, sucrose, xylitol, sorbitol, galactose and maltose. The nitrogen sources optimised include ammonia sulphate, ammonia chloride, acrylamide, magnesium nitrate and urea. The pH ranging from 4 to 8 was optimised using appropriate buffers. Varied pH was adjusted by different overlapping buffers system including 50 mM acetate buffer for pH 4.0, 5.0, and 6.0, 50 mM Tris buffer pH 6.0 and 7.0; 50 mM Tris-HCl pH 7.0 and 8.0. Glyphosate tolerance was carried out in the range of 50–400 ppm. All parameters involving carbon sources, nitrogen sources, pH, temperature and glyphosate concentration were carried out within a timeframe of 48 h while maintaining the same inoculum size of 2% (w/v).

Data analysis using response surface methodology (RSM)

Two stages of optimisation of glyphosate-degrading bacteria were done using response surface methodology. The first stage was done by Plackett–Burman (PB) followed by central composite design (CCD). All experiments were carried out in triplicates with the average taken as the response. Percentage of glyphosate degradation was used as a response. The correlation of the response and independent variables was specified using the second order model that suits the response variable. The universal formula for the 2nd degree polynomial model used is as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1 + \beta_{22} X_2 + \beta_{33} X_3 + \beta_{44} X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{14} X_1 X_4 + \beta_{34} X_3 X_4,$$

where parameter Y is the predicted response and β_0 , β_1 , β_2 , β_3 , β_4 , β_{11} , β_{22} , β_{33} , β_{44} , β_{12} , β_{13} , β_{23} , β_{24} , β_{14} and β_{34} are constant regression coefficients of the model in which β_0 is the intercept term, β_1 , β_2 , β_3 , and β_4 are linear coefficients

and β_{11} , β_{22} , β_{33} and β_{44} are the squared coefficients. On the other hand X_1 , X_2 , X_3 and X_4 are the independent parameters. Parameters grouping (X_1 , X_2) show the interaction among the unpredictable (Karamba et al. 2016). The significant factors that were identified using PB were further optimised using CCD. The design of the experiment and the statistical analysis of data were conducted using Design Expert software version 6.0.8.

Statistical analysis

All experiments were conducted in triplicate. Experiments errors were shown in error bars format as standard deviation indicating in three determinations. All data were statistically analysed using GraphPad v3.5. A one-way ANOVA (95% confidence interval) and t test (LSD) for biodegradation were utilised to appraise the difference among parameters with $p < 0.05$ considered as statistically significant.

Results and discussion

Effects of carbon source

The ability of thirteen different carbon sources to promote the degradation of glyphosate was studied in this experiment (Fig. 1). Fructose obtained the highest effect of aiding glyphosate degradation with 83.6% removal in the medium and bacterial growth rate of 3.43×10^8 CFU/mL compared to other carbon sources. Glucose showed 79.7% of glyphosate degradation, followed by sucrose with 75.7% degradation and rhamnose with 70.1% glyphosate degradation. Other carbon sources indicated the least effect in assisting degrading by removing less than 50%. No bacterial growth or glyphosate degradation was observed when glycerol is used as the carbon source. In view of this effect, fructose was considered as the most suitable carbon source for further the study on optimisation, although it showed no significant difference with glucose. This is because *Burkholderia* spp. is usually found in sugarcane roots that promote plant growth which is rich in fructose and sucrose (Paungfoo-Lonhienne et al. 2014). Acclimation to this fructose rich environment has led toward more efficiency in degrading glyphosate. Moreover, *B. vietnamiensis* strain AQ5-12 was isolated from sugarcane plantation, therefore, illustrated high affinity towards fructose with highest degradation of glyphosate compared to glucose (Manogaran et al. 2017). *Pseudomonas fluorescens* and *Acetobacter* sp. were able to efficiently utilise glyphosate when glucose is introduced to the medium (Moneke et al. 2010). Gluconate and pyruvate were used as carbon source by *Flavobacterium* sp. (Balthazor and Hallas 1986). Sodium glutamate was used as the carbon source for studying the degradation of glyphosate by *Ochrobactrum* sp.

Fig. 1 Effects of different carbon source on biodegradation of 50 ppm glyphosate by resting cells of *Burkholderia vietnamiensis* isolate AQ5-12. Error bars represent mean $\pm$ standard deviation ($n = 3$)

strain GDOS (Hadi et al. 2013). However, there is lack of literature available on the effects of different carbon source on biodegradation of glyphosate since most of the reports only utilised glucose as a carbon source.

Effects of fructose concentrations

Figure 2 displays the effects of different fructose concentration on glyphosate degradation to determine the best condition for optimum removal rate. Fructose concentration from 1 to 10 g/L was used in this study. Increasing fructose concentration resulted in increased glyphosate degradation. A fructose concentration of 0.6 g/L aided glyphosate degradation by 86.3% with bacterial growth of $3.35E + 08$ CFU/mL. *T* test analysis showed no significant difference between 6, 7, 8, 9 and 10 g/L ($p > 0.05$) although the highest degradation of glyphosate was observed at 7 g/L with 89.3%, followed by 88.7% degradation at 8 g/L and 87.8% degradation at

9 g/L. Therefore, it can be concluded that increasing fructose concentration beyond 6 g/L gave no significant difference in degradation rate. Thus, 6 g/L fructose concentration was used for the rest of experiments.

Effects of nitrogen source

Five different nitrogen sources which comprise both organic and inorganic nitrogen sources were examined in this study. For each nitrogen source, 0.5 g/L of concentration was used with 50 ppm of filter-sterilised glyphosate for 24 h as in Fig. 3. Magnesium nitrate and ammonia sulphate showed degradation of 85.7 and 81.30% with bacterial growth of $3.37E + 08$ and $3.28E + 08$ CFU/mL, respectively. Both nitrogen sources demonstrated a significant difference ($p < 0.05$) with mean difference of 4.4 when glyphosate is used as a sole phosphorus source. Meanwhile, ammonia chloride and acrylamide were seen

Fig. 2 Effects of different fructose concentration on biodegradation of 50 ppm glyphosate by resting cells of *Burkholderia vietnamiensis* strain AQ5-12. Error bars represent mean $\pm$ standard deviation ($n = 3$)

Fig. 3 Effects of nitrogen source on biodegradation of 50 ppm glyphosate by resting cells of *Burkholderia vietnamiensis* strain AQ5-12. Error bars represent mean $\pm$ standard deviation ($n = 3$)

as the poor sources of nitrogen as they can only assist the degradation by 51.23 and 39.93%, respectively. Least degradation was observed when urea is supplemented as nitrogen source with degradation of 15.42%. Ammonia chloride aided glyphosate degradation much better compared to acrylamide and urea with a significant difference ($p < 0.001$). In control, which had no nitrogen source, showed a depletion of 5.8% glyphosate with a bacterial growth of 2.58×10^7 CFU/mL. Like any nitrogen fixing bacteria, *Burkholderia* spp. are capable of transforming atmospheric nitrogen into fixed nitrogen (Santos et al. 2001). This allowed *B. vietnamiensis* strain AQ5-12 to proliferate and degrade glyphosate by taking the advantage of this mechanism. Furthermore, glyphosate contains nitrogen in its chemical composition (Duke and Powles 2008). Therefore, the utilisation of such compound as a source of nitrogen is the exception. *Arthrobacter* sp. strain GLP-1 is one of the earliest reported strain able to utilise glyphosate as sole nitrogen source (Pipke and Amrhein 1988). In choosing the best nitrogen source for optimal glyphosate degradation, ammonia sulphate was selected over magnesium nitrate despite its slightly having better degradation rate compared to ammonia sulphate. This is because ammonia sulphate is more cost effective than magnesium nitrate which can minimise the overall cost for the study (Soletto et al. 2005). Moreover, unwanted excess ammonia sulphate that is toxic to the environment can be used for a mass production of glyphosate-degrading enzyme using strain AQ5-12 rather than disposing it away, thus making it an environmentally friendly process (Boyd and Massaut 1999).

Effects of ammonia sulphate concentration

Figure 4 shows the effects of various ammonia sulphate concentration on degrading 50 ppm glyphosate by *B.*

vietnamiensis strain AQ5-12. Increasing ammonia sulphate concentration of up to 0.6 g/L showed an increment in glyphosate degradation. Highest degradation of glyphosate was observed at 0.6 g/L by 85.1% with bacterial growth of 3.38×10^8 CFU/mL. At 0.7 g/L, glyphosate degradation was found to be 83.1% with bacterial growth of 3.32×10^8 CFU/mL, illustrating an increase beyond 0.6 g/L results in lower degradation of glyphosate. At 0.5 g/L, glyphosate degradation was 81.9%. The lowest bacterial growth was observed at 0.1 g/L with 2.0×10^8 CFU/mL with degradation of 50.1%. Although ammonia sulphate concentration of 0.6 g/L resulted highest glyphosate degradation rate, there was no significant difference in degradation between 0.5, 0.6 and 0.7 g/L ($p > 0.05$) based on ANOVA analysis. The use of 0.5 g/L of ammonia sulphate will provide same degradation result as using 0.6 or 0.7 g/L. Thus, using 0.6 g/L of ammonia sulphate will be a waste of resource and inefficient. 0.5 g/L of ammonia sulphate concentration was used for the rest of the experiments.

Effects of pH

Figure 5 presents the effects of pH on bacterial growth and degradation of 50 ppm glyphosate by *B. vietnamiensis* strain AQ5-12. pH maintenance is very crucial in bacterial medium as it affects the growth and proliferation of cells since overlapping buffer were used, only highest degradation were discussed at pH 6 and 7. Acidic pH of 4, 4.5 and 5 illustrated low biodegradation on glyphosate. Lowest degradation was observed at pH 4 with the removal of 25% and bacterial growth of 2.86×10^8 CFU/mL, followed by pH 4.5 with degradation of 46.67% and bacterial growth of 2.95×10^8 CFU/mL. Optimum degradation occurs at pH 6–7.5 where pH 6.5 illustrated the highest degradation of glyphosate by 84.67% followed by pH 7 with 84.23% degradation. ANOVA analysis showed no significant difference

Fig. 4 Effects of different ammonia sulphate concentrations on biodegradation of 50 ppm glyphosate by resting cells of *Burkholderia vietnamiensis* strain AQ5-12. Error bars represent mean $\pm$ standard deviation ($n = 3$)

Fig. 5 Effects of pH on bacteria growth (filled blue circle, red square, black diamond) and biodegradation (open blue circle, red square, black diamond) of 50 ppm glyphosate by resting cells of *Burkholderia vietnamiensis* strain AQ5-12. Acetate buffer (filled blue circle,

open blue circle), Tris (filled red square, open red square), Tris-HCl (filled black diamond, open black diamond). Error bars represent mean $\pm$ standard deviation ($n = 3$)

in degradation rate between pH 6.5 and 7 with a mean difference of 0.94 ($p > 0.05$), although pH 6.5 showed the highest degradation compared to other pH variables. pH 7 illustrated a relatively good biodegradation rate compared to pH 8. pH 8 has enhanced glyphosate removal by 66.76% with bacterial growth of $3.07E + 08$ CFU/mL. *Burkholderia vietnamiensis* strain AQ5-12 showed an improvement in degradation of glyphosate by 11.3% at pH 6 compared to pH 8, suggesting that a slightly acidic condition is preferable for better degradation of glyphosate compared to the alkaline condition. At pH 6, Tris buffer aided biodegradation of glyphosate much better

compared to acetate buffer; therefore, Tris buffer was used subsequently for pH 6. Both Tris and Tris-HCl buffer showed similar biodegradation results at pH 7. *Arthrobacter* sp. strain GLP-1 (Pipke and Amrhein 1988) and *Bacillus cereus* CB4 (Fan et al. 2012) showed optimum glyphosate degradation at pH 6.0–7.0. More alkaline condition was preferred by *Pseudomonas putida* (Benslama and Boulahrouf 2013) for optimum growth. However, inadequate literature is available on glyphosate-degrading bacteria favouring acidic condition since most glyphosate degraders prefer neutral-alkaline pH for optimum degradation rate (Singh and Walker 2006).

Fig. 6 Effects of temperature on biodegradation of 50 ppm glyphosate by resting cells of *Burkholderia vietnamiensis* strain AQ5-12. Error bars represent mean $\pm$ standard deviation ($n = 3$)

Effects of temperature

Figure 6 displays the effects of temperature on the biodegradation of 50 ppm glyphosate and bacterial growth of *B. vietnamiensis* strain AQ5-12. Temperature conditions were optimised in an array of 25–60 °C. The optimum glyphosate degradation occurred from 30 to 35 °C, which illustrated a degradation of at least 80%. The excellent temperature demonstrated by this bacterium in both biodegradation and growth was 32 °C, which has improved the biodegradation of glyphosate by 85.83% with bacterial growth of $3.5\text{E} + 08$ CFU/mL. The temperature of 30 °C was second with a biodegradation enhancement of 83.16% and a growth rate of $3.41\text{E} + 08$ CFU/mL, followed by 35 °C with degradation of 82.9% with bacterial growth of $3.43\text{E} + 08$ CFU/mL. ANOVA analysis showed no significant difference between 32 and 35 °C ($p > 0.05$) with a mean difference of 2.267; however, both temperatures showed significant difference against 37 °C ($p < 0.01$). Other temperature conditions of 37 and 40 °C demonstrated biodegradation rate of 72.3 and 66.67%, respectively, with proliferation rate of the bacterium abating in the declining order of biodegradation, showing a growth rate of $3.25\text{E} + 08$ and $3.01\text{E} + 08$ CFU/mL, respectively. Although the temperature of 25 °C showed the lowest bacterial growth of $2.67\text{E} + 08$ CFU/mL, it illustrated 5.47% more degradation of glyphosate compared to 40 °C. No bacterial growth and degradation were observed at 50 and 60 °C. Several bacteria have been reported to degrade glyphosate at various temperatures, but the preferable temperature is 30 and 35 °C. *Ochrobactrum* sp. GDOS (Hadi et al. 2013) and *P. putida* (Benslama and Boulahrouf 2013) reported the highest bacterial growth at 30 °C, suggesting extensive utilisation of glyphosate by these bacteria. Meanwhile, *B. cereus* CB4 showed optimum degradation at 35 °C (Fan et al. 2012). To date, *Geobacillus*

caldoxylosilyticus T20 is the only bacterial strain capable of utilising glyphosate as phosphorus at 60 °C (Obojska et al. 2002).

Effects of glyphosate concentration

Figure 7 demonstrates the effects of different glyphosate concentration on its degradation and bacterial growth rate *B. vietnamiensis* strain AQ5-12. The ability for *B. vietnamiensis* strain AQ5-12 to degrade a high concentration of glyphosate was tested to ascertain the optimum concentration of glyphosate that can able to degrade by the strain. An array of 50–400 ppm glyphosate was utilised in this study. Highest degradation was recorded in the presence of 100 ppm, which stood at 86.4% with bacterial growth of $3.78\text{E} + 08$ CFU/mL. The 50 ppm glyphosate concentration was second with a biodegradation enhancement of 85.37% and a growth rate of $3.42\text{E} + 08$ CFU/mL, followed by 150 ppm degradation by 79.1% with bacterial growth rate of $3.36\text{E} + 08$ CFU/mL. Based on ANOVA analysis, there was no significant difference ($p > 0.05$) in degradation between 50 and 100 ppm of glyphosate concentration with a mean difference of -2.040 . Significant difference was observed between 100 and 150 ppm ($p < 0.05$). Increasing glyphosate concentration beyond 100 ppm illustrated a decrease in bacterial growth, which resulted in poor degradation rate. Lowest degradation of glyphosate was observed at 400 ppm with the rate of 49.4%, which had almost reduced its biodegradation capability by half compared to 100 ppm. It was reported that several bacteria are able to degrade glyphosate at various concentrations. *Acetobacter* sp. and *P. fluorescens* were reported to have an optimal growth at 7500 ppm with both strains able to tolerate up to 250,000 ppm of glyphosate (Moneke et al. 2010). 20 mM of glyphosate significantly inhibited the growth of *P. oryzae* despite having glucose as another carbon source (Kuklinsky-Sobral et al.

Fig. 7 Effects of glyphosate concentration on biodegradation of glyphosate by resting cells of *Burkholderia vietnamiensis* strain AQ5-12. Error bars represent mean $\pm$ standard deviation ($n = 3$)

2005). *Bacillus cereus* was found with a capacity to utilise herbicide glyphosate at optimum concentration of 6000 ppm glyphosate (Fan et al. 2012).

Data analysis with RSM using Plackett–Burman (PB) and central composite design (CCD)

Five independent parameters were screened with 12 different combinations of experiment to identify significant factor(s). Each parameter comprised low level (-1) and high level ($+1$), which is the minimum and maximum values ran during OFAT phase. Design Expert software version 6.0.8 was used for the regression analysis of the acquired experimental data to plot response surface in optimising significant factors. The ranges for these five variables affecting glyphosate degradation are as follow: glyphosate concentration: 50–400 ppm, carbon source: 1–10 g/L, nitrogen source: 0.1–0.7 g/L, temperature: 25–40 °C and pH 4–8. A total of

12 experiments were conducted in PB using state parameters as shown in Table 1. The result obtained for percentage removal was taken as the response and used for statistical analysis. Meanwhile, significant variables found were further subjected to optimisation using central composite design.

The Model F value of 144.91 implies the model is significant, which was obtained from ANOVA analysis using the factorial design (Table 2). There is only a 0.01% chance that a “Model F -Value” this large could occur due to noise. Values of “Prob $> F$ ” < 0.0500 illustrates that model specifications are significant. In this stipulation, carbon concentration and pH were considered as significant model provisions. Therefore, these two parameters were utilised in designing CCD. The ANOVA displayed an R^2 of 0.9943, which is the data highly fitted in regression line. The “Pred R -Squared” of 0.9420 is in reasonable agreement with the “Adj R -Squared” of 0.9874, which

Table 1 Experimental design and results of Plackett–Burman on glyphosate degradation by different parameters of carbon, nitrogen, glyphosate, pH and temperature

Run	A	B	C	D	E	Actual value	Predicted value
1	1	0.7	4.0	25	50	41.00	37.86
2	1	0.1	4.0	40	400	56.00	57.29
3	10	0.1	4.0	25	400	46.40	52.35
4	1	0.7	8.0	40	50	92.10	93.44
5	10	0.1	8.0	40	50	94.50	93.05
6	1	0.7	8.0	25	400	55.00	52.35
7	10	0.7	4.0	40	50	35.70	37.00
8	10	0.7	4.0	40	400	45.40	45.25
9	10	0.1	8.0	25	50	92.90	93.05
10	1	1.0	4.0	25	50	36.00	37.86
11	10	0.7	8.0	25	400	39.50	40.80
12	1	0.1	8.0	40	400	51.00	52.35

A carbon concentration, B nitrogen concentration, C pH, D temperature (°C), E glyphosate concentration

Table 2 Analysis of parameters for glyphosate degradation for Plackett–Burman by different parameters of carbon, nitrogen, glyphosate, pH and temperature

Source	Sum of squares	df	Mean square	F value	Prob > F	
Model	5641.45	6	940.24	144.91	< 0.0001	Significant
A	102.95	1	102.92	15.86	0.0105	
C	1741.83	1	1741.83	268.45	< 0.0001	
E	718.32	1	718.32	110.71	0.0701	
AC	2441.38	1	2441.38	376.26	< 0.0001	
AE	83.52	1	83.25	12.83	0.1158	
CE	0.16	1	0.016	0.024	0.8825	
Residual	32.44	5	6.49			
Cor total	5673.90	11				
Std. dev	2.55		R^2		0.9943	
Mean	57.13		Adj R^2		0.9874	
C.V	4.46		Pred R^2		0.9420	
PRESS	329.20		Adeq precision		29.011	

Table 3 Central composite experimental design for glyphosate degradation

Run	A	B	Actual	Predicted
1	0.86	6.00	62.10	62.31
2	5.50	3.17	89.00	87.77
3	5.50	6.00	88.60	87.42
4	10.00	4.00	92.00	93.28
5	5.50	8.83	73.40	74.10
6	1.00	4.00	74.00	92.26
7	5.55	6.00	87.30	87.42
8	5.50	6.00	87.10	87.20
9	5.50	6.00	89.00	87.42
10	1.00	8.00	63.20	62.44
11	10.00	8.00	86.20	87.42
12	11.86	6.00	93.00	92.26
13	5.50	6.00	85.00	87.42

A carbon concentration (fructose), B pH

clarifies the impact of the model. The adeq precision was obtained to be 29.011, indicating an acceptable and satisfactory signal.

Central composite design (CCD)

A total of 13 experiments were resulted from CCD design with their responses shown in Table 3. Based on ANOVA analysis, the coefficients determination, R^2 of the model was found to be 0.9887, whereas the adjusted R^2 was found to be 0.9807 showing a high correlation between the experimental design data (Table 4). Moreover, the high R^2 value was seen able to clarify up to 98.9% discrepancy of the response. This evaluates how inconsistency studied response value can be clarified by research parameters and their relations. The adjusted R^2 value was inferred at 0.9807, which is a well satisfactory rate. The significance of this polynomial model was clarified. The “Pred R-Squared” of 0.9607 is in coherent

Table 4 ANOVA analysis for central composite design

Source	Sum of squares	df	Mean square	F value	Prob > F	
Model	1315.51	5	263.10	122.79	< 0.0001	Significant
A	896.72	1	896.74	418.51	< 0.0001	
B	186.84	1	186.84	87.20	< 0.0001	
A ²	178.55	1	178.55	45.76	< 0.0001	
B ²	73.08	1	73.08	34.11	0.0006	
AB	6.25	1	6.25	2.92	0.1314	
Residual	15.00	7	2.14			
Lack of fit	5.19	3	1.73	0.71	0.5967	Not significant
Pure error	9.81	4	2.45			
Cor total	1330.51	12				
Std dev.	1.46		R^2		0.9887	
Mean	82.31		Adj R^2		0.9807	
C.V.	1.78		Pred R^2		0.9607	
PRESS	52.24		Adeq precision		31.141	

agreement with the “Adj R -Squared” of 0.9807. A ratio of > 4 was required, which indicates an acceptable signal. Full term precision was obtained as 31.141. Adeq precision measured the signal to noise ratio. Ratio greater than 4 is considered desirable. The significance of all coefficients was ascertained by p values. Test for lack of fit is demonstrated in Table 4. In addition to make all the model terms to be significant, lack of fit of the model was found to be insignificant, which is strongly desirable for the adequate model. The lack of fit test denotes whether the selected model is adequate to describe the observed data or a more complicated mode is required. The model F value of 122.79 indicates that the model is significant. Moreover, there is only a 0.01% possibility that a “Model F -Value” this large could occur because of noise. The value of $\text{Prob} > F < 0.0500$ illustrated that A , B , A^2 , B^2 and AB are significant model terms. The coefficient of variation for the experiment was 4.46 (less than 10 to be good) showing the experiments were reproducible. Therefore, the final equation in terms for coded factors is shown below.

Final equation in terms of coded factors:

$$\text{Degradation (\%)} = +87.42 + 10.59 \times A - 4.83 \times B - 5.07 \times A^2 - 3.24 \times B^2 + 1.25 \times A \times B.$$

Final equation in terms of actual factors:

$$\begin{aligned} \text{Degradation (\%)} = & +56.82198 + 4.27146 \times \text{carbon conc.} \\ & + 6.54350 \times \text{pH} - 0.25019 \times \text{carbon conc.}^2 \\ & - 0.81031 \times \text{pHd} + 0.13889 \times \text{carbon conc.} \times \text{pH}. \end{aligned}$$

The similarity between the predicted and the actual assessment of the study on response accomplished is illustrated in Fig. 8. The plot shows a close correlation of both predicted and actual data as data points from the gathered close to the bisection line that divides the plot into equal 45° halves. It indicated that the responses are close to the

predicted values by the experimental design which results in high similarity between them.

Each 3D response surface graph plotted for glyphosate degradation represents different combinations of the two parameters (X_1 ; X_2 ; X_3 ; X_4) at one time while maintaining the other parameters at zero level (constant).

Figure 9 describes the effects of carbon concentration (X_1) and pH (X_2) on glyphosate degradation while keeping glyphosate concentration at 50 ppm, temperature at 32°C and nitrogen concentration at 0.6 g/L. The high percentage of glyphosate degradation was observed within carbon concentration range (8.0–10.0 g/L) and pH range (4–6). Furthermore, the optimum level of carbon concentration at 10 g/L and pH 4 showed the maximum percentage of glyphosate degradation of up to 93.28%. Low level of degradation was observed at pH 8 degrading at 62.44%, whereas pH 4 degrading at 74.4% when carbon concentration remained constant at 1 g/L. *Burkholderia vietnamiensis* strain AQ5-12 was observed to degrade better in acidic condition compared to the alkaline condition in a low carbon environment. *Burkholderia vietnamiensis* strain AQ5-12 was able to degrade 93.28% glyphosate at pH 4 compared at pH 8 when carbon concentration is increased to 10 g/L where it degrades 7.59% lesser. The half elliptical shape of surface and contour plot indicated that the mutual interaction between the two independent factors (X_1 , X_2) was significant. However, to further verify whether increasing carbon concentration beyond 10 g/L while maintaining pH at 4 have any significant improvement on glyphosate degradation, additional experiment was conducted to validate the results.

Based on the 3D plot (Fig. 9) the RSM illustrated higher degradation of glyphosate in 10 g/L fructose at pH 4. This shows there was a possible improvement in degradation rate if the concentration of carbon source is increased. Figure 10 shows the effects of increasing fructose concentration to 100 ppm on glyphosate degradation while maintaining

Fig. 8 Similarity plot between predicted and actual value for free cells of *Burkholderia vietnamiensis* strain AQ5-12

Fig. 9 Response surface 3D plot showing the effect of pH and carbon concentration on glyphosate degradation. Value variable temperature, nitrogen and glyphosate concentration remained constant

DESIGN-EXPERT Plot

Utilisation
X = A: carbon
Y = B: pH

Fig. 10 Effects of higher fructose concentration on biodegradation of 100 ppm glyphosate by resting cells of *Burkholderia vietnamiensis* strain AQ5-12. Error bars represent mean $\pm$ standard deviation ($n = 3$)

pH at 4, the temperature at 32 °C and ammonia sulphate at 0.5 g/L. 11 g/L of fructose concentration illustrated the highest degradation, which stood at 91.7% with bacterial growth of $3.88E + 08$ CFU/mL. An increase in fructose concentration to 12 g/L resulted in 90% degradation with the bacterial growth of $3.52E + 08$ CFU/mL. Concentrations beyond 13 g/L showed a linear decrease in glyphosate degradation when bacterial growth was significantly reduced. The optimum level of carbon concentration 10 g/L and pH level 4 showed maximum percentage of glyphosate degradation of up to 93.28% based on RSM results. In comparison, the results from RSM (Fig. 9) and effect of carbon source beyond 11 g/L (Fig. 10) with a degradation of 93.28 and 91.7%, respectively, had no significant difference between them ($p > 0.05$) if the pH is maintained at 4. The additional

increase in carbon concentration beyond 12 g/L has significantly reduced biodegradation of glyphosate. Therefore, it can be concluded that increasing fructose concentration beyond 11 g/L while maintaining pH at 4 give no significant effect on degradation rate by *B. vietnamiensis* strain AQ5-12.

Figure 11 shows the validation of experiment based on condition provided by RSM for optimum degradation of glyphosate. Based on the solution provided by CCD, the factors was 8.62 g/L of fructose and pH of 5.41, while the remaining factors were maintained at constant based on OFAT results since PB analysis excluded them due to no significant interaction shown between the variables. RSM predicted a degradation rate of 97.723% of 100 ppm of glyphosate. The confirmation experiment was organised with the optimal factors obtained from the response. An

Fig. 11 Validation of experiment (RSM) conducted to ascertain the optimum solution obtained through the conduct of the experiment. Error bars represent mean $\pm$ standard deviation ($n = 3$)

Table 5 Comparison of optimum conditions and results obtained between OFAT and RSM

Conditions	OFAT	RSM
Carbon (g/L)	6	8.62
Nitrogen (g/L)	0.5	0.5
pH	6.5	5.41
Temperature (°C)	32	32
Glyphosate (ppm)	100	100
Degradation	86.367	93.723%

experiment was conducted to verify this result using the suggested solution provided by CCD. Based on the provided solution by RSM (Table 5), *B. vietnamiensis* strain AQ5-12 has degraded 92.32% of 100 ppm glyphosate in a time frame of 24 h, which displayed high similarity with the predicted degradation rate (Fig. 11). ANOVA analysis showed no significant difference among the results obtained in the RSM and the validation of results carried out. This is evidently in close agreement with the model prediction. Temperature, nitrogen and glyphosate concentration showed similar values in OFAT and RSM, whereas minor changes were obtained on the amount of concentration for carbon and pH, thus resulting in better degradation. By increasing carbon source by 2.62 g/L and reducing pH by 1.09, 7.35% more degradation rate was compared to OFAT result. In comparison, RSM resulted in better degradation condition compared to OFAT condition (Table 5).

Conclusion

Combination of 8.62 g/L fructose concentration, 0.5 g/L ammonia sulphate concentration, pH 5.41, temperature 32 °C and 100 ppm glyphosate concentration provided

optimal glyphosate degradation rate by *B. vietnamiensis* strain AQ5-12. Further increase in concentrations revealed that the strain could tolerate up to 400 ppm of glyphosate. There is a close correlation between the optimum parameters used for both OFAT and RSM. Fructose and pH show a difference of 2.62 g/L and 1.09, respectively. The temperature, ammonia sulphate and glyphosate concentration remained the same. However, the difference in degradation differs significantly, as for OFAT the maximum concentration degraded was 86.37% of 100 ppm, while RSM it was 92.32%. Therefore, it can be summarised that the RSM with central composite design can be used for optimising variables needed for the enhancement of glyphosate biodegradation by *B. vietnamiensis* strain AQ5-12.

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