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Optimisation of Culture Conditions for PLA-food-packaging Degradation by *Bacillus* sp. SNRUSA4

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ABSTRACT

Polylactic acid (PLA) is increasingly used in food-packaging production. The screening of PLA-food-packaging-degrading bacteria and optimisation of culture conditions for the PLA-food-packaging degradation by PLA-food-packaging-degrading bacteria were investigated for bioplastic waste management purposes. Only bacterial strain SNRUSA4 exhibited an increase in optical density (OD) in Basal Medium (BM) supplemented with 1.0 g/L of PLA-food-packaging as sole carbon source after 4 weeks of incubation. A weight loss of 7.3% and the rough and porous surface of PLA-food-packaging indicated that SNRUSA4 was a PLA-food-packaging-degrading bacterium. SNRUSA4 was able to degrade pure PLA which was confirmed from the clear zone formation around its colony on emulsified pure PLA agar plate. The 16S rRNA gene sequence of SNRUSA4 showed the similarity with thirteen *Bacillus* species. Hence, the strain SNRUSA4 was assigned as *Bacillus* sp. SNRUSA4. Response surface methodology with Box-Behnken Design was used to optimise the culture conditions including yeast extract concentration, initial pH value, temperature and agitation speed for growth and PLA-food-packaging degradation of *Bacillus* sp. SNRUSA4. The optimal conditions of *Bacillus* sp. SNRUSA4 was discovered in BM at initial pH value 7.02 with yeast extract concentration of 2.56% and agitated at 205.28 rpm at 31.68°C. Under optimal conditions, the OD of *Bacillus* sp. SNRUSA4 was up to 1.955, and the different OD between before and after optimisation was up to 1.752. Furthermore, the PLA-food-packaging weight loss also increased from 7.30% to 87.10% indicating that the

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PLA-food-packaging degradation under optimal conditions was higher than the unoptimised conditions. Therefore, *Bacillus* sp. SNRUSA4 is an efficient strain for degradation of PLA and PLA-food-packaging.

Keywords: *Bacillus* sp. SNRUSA4, biodegradation, Box-Behnken design, response surface methodology

INTRODUCTION

Plastics made from petroleum such as polyamides (nylon), polyethylene, polystyrene, and poly(vinyl chloride). are non-degradable materials which lead to the accumulation of plastic wastes in environment. Polylactic acid (PLA) is considered as an alternative plastic to reduce the environmental problem. Lactic acid (monomer of PLA) was produced through the fermentation of renewable resources and biodegradable materials (Tawakkal, et al., 2014; Muller et al., 2017). PLA is a nontoxic compound and is accepted as generally recognized as safe by FDA (Food and Drug Administration) (Tawakkal et al., 2014). PLA-food-packaging is widely used in drinking cups (Farah et al., 2016) and various food grade containers (Zhong et al., 2020). The production of PLA had increased from 140,000 tonnes per annum in 2011 to 800,000 tonnes per annum in 2020 (Mirabal et al, 2013). The PLA-food-packaging degradation depends on the environmental conditions. The degradation of PLA in natural condition (soil and sludge) spends more than 90 days (Boonmee et al., 2016). The usage rate of PLA-food-packaging is greater than the PLA degradation rate, potentially causing additional environmental problems in the future.

The PLA degradation occurs by cleavage of ester bonds between two lactic acid molecules. Hydrolytic (Elsawy et al., 2017), photolytic (Janorkar et al., 2007) and microbial degradation (Apinya et al., 2015; Liang et al., 2016; Lipsa et al., 2016; Bubpachat et al., 2018) are three recognised mechanisms of PLA degradation. Microbial degradation is one of the interesting topics of PLA degradation research, as the complete degradability of PLA does not cause any pollution to the environment (Qi et al., 2017). Karamanlioglu et al. (2014) demonstrated that PLA-food-packaging did not degrade in sterile soil and suggested that microorganisms as biocatalyst could help to degrade PLA-food-packaging. Microbial degradation of PLA-food-packaging by the two fungi, Aspergillus ustus and Penicillium verrucosum was first found by Szumigaj et al. (2008). Streptomyces sp. KKU215 is the first actinomycete strain for PLA-food-packaging degradation (Yottakot & Leelavatcharamas, 2019). There have been no reports on PLA-food-packaging degradation by unicellular bacteria. Thus, isolation and screening of microorganisms capable of degrading PLA-food-packaging is an interesting research.

The utilisation of Response Surface Methodology (RSM) based on Box-Behnken Design (BBD) for the optimisation of culture conditions is the currently accepted method (Khatoon & Rai, 2020). This statistical technique can provide the information of interaction between parameters (Qi et al., 2015). Moreover, the degradation of PLA and PLA-food-

packaging has been significantly increased through the use of RSM with BBD (Chaisu et al., 2012; Yottakot & Leelavatcharamas, 2019).

Therefore, the aim of this research was to screen, identify and optimise culture conditions of PLA-food-packaging-degrading bacteria. RSM with BBD was used to determine the optimal culture conditions (concentration of yeast extract (*YE*), initial pH value (pH_{ini}), temperature (*T*) and agitation speed (*AS*)) of *Bacillus* sp. SNRUSA4 growth for PLA-packaging degradation.

MATERIALS AND METHODS

Screening of PLA-food Packaging-Degrading Bacteria

PLA-food-packaging was purchased from Dairy Home Co., Ltd, Thailand. PLA-food-packaging was cut into samples of size 1 cm x 1 cm, then PLA-food-packaging coupons was washed with 70% (v/v) ethanol and allowed to air-dry until completely dry. The samples were collected from soils and composts to isolate the PLA-packaging-degrading bacteria. These samples were kept at 4°C until required.

The sterile Basal Medium (BM) (pH 7.0) was prepared by adding 4 g of $(NH_4)_2SO_4$, 2 g of K_2HPO_4, 2 g of KH_2PO_4 and 0.5 g of $MgSO_4.7H_2O$ to 1 L of distilled water, and then autoclaved at 121°C for 15 minutes. The sterile BM was added 1 g of PLA-food-packaging coupons as sole carbon source.

In order to isolate PLA-food packaging degrading bacteria, 10 g of the samples was inoculated into 250 mL Erlenmeyer flask with 100 mL of sterilised BM containing 1 g of PLA-food-packaging coupons. The inoculated flask was incubated at 37°C and 180 rpm in an incubator shaker for 7 days. After incubation, 10 mL of the culture was inoculated into sterilised BM containing 1 g of PLA-food-packaging coupon as sole carbon source. This experiment was performed five times with the same method. Then, each of the PLA-food-packaging coupons was transferred onto BM agar plates. The plates were incubated at 37°C for 7 days (Yottakot & Leelavatcharamas, 2019). The colonies around PLA-food-packaging coupons were purified by streak plate technique on Nutrient Agar (NA) plate. The purified isolates were stored on NA slant at 4°C and in 30% glycerol at -10°C.

PLA-food packaging-degrading bacteria was screened by analysing the growth in the sterile BM containing 1 g of PLA-food-packaging coupons as sole carbon source using the optical density (OD) at wavelength 600 nm (Lee et al., 2013). The cell suspension of selected stains was transferred to Nutrient Broth (NB) medium and shaken at 180 rpm at 37°C for 24 hours, and then centrifuged for 10 min at 5,635 x g. The precipitated cells were washed twice with 0.85% (w/v) NaCl. The cell density was adjusted to OD_{600} = 1.0 with 0.85% (w/v) NaCl. The optical density was measured by spectrophotometer. Cell suspension (10 mL) of each isolate was inoculated into 100 mL of the sterile BM containing 1 g of PLA-food-packaging coupons as sole carbon source, and then incubated at 37°C and 180

rpm in an incubator shaker. The cell density was measured every week for 4 weeks. The sterilised BM, BM containing 1 g of PLA-food packaging coupons and BM with the cell were used as controls. The experiment was done in triplicate.

SNRUSA4, PLA-food-packaging-degrading bacterial strain was screened because of the increasing OD. The ability of SNRUSA4 to degrade PLA-food-packaging coupons was measured after 4 weeks of incubation by analysing weight loss and surface changes of the individual samples in the sterilised BM containing 1 g of PLA-food-packaging coupons as sole carbon source. The original PLA-food-packaging coupons and PLA-food-packaging coupons in BM without inoculation of SNRUSA4 after 4 weeks of incubation were used as controls.

Degradation of PLA-food-packaging coupons was assessed by measuring weight loss. The PLA-food-packaging coupons was washed with distilled water, and dried until constant weight in an electronic desiccator. The weight loss percentage of PLA-food-packaging coupons was calculated according to Equation 1 (Vey et al., 2007).

$$\%\, Weight\ loss = \frac{m_{ini} - m_{dry}}{m_{ini}} \times 100\% \qquad [1]$$

where m_{ini} is the initial weight of original PLA-food-packaging coupon; m_{dry} is the dry weight of PLA-food-packaging coupon after degrading.

The change in surface morphology of the PLA-food-packaging coupon was checked after 4 weeks of incubation by Scanning Electron Microscope (SEM) (SEC, model SNE-4500M). The PLA-food-packaging coupons were coated with gold before SEM examination.

The pure PLA degradability of PLA-food-packaging-degrading bacterial strain SNRUSA4 was confirmed by the clear zone around bacterial strain SNRUSA4 colony on emulsified PLA agar plate after 2 weeks of incubation at 37°C. PLA pellet (2003D grade, melting temperature 210°C, average molecular weight 200,000 g/mol) was obtained from NatureWorks LLC (U.S.A.). Emulsified PLA agar was prepared as follows by the method of Yottakot & Leelavatcharamas (2019).

Identification of Selected Strain

The PLA-food-packaging-degrading bacterial isolate SNRUSA4 was identified on the basis of 16S rRNA gene sequence analysis. DNA of SNRUSA4 was extracted using DNA mini kit (Geneaid Biotech Ltd., Taiwan). The 16S rRNA gene was amplified from the genomic DNA by PCR using the two primers, 20F and 1500R (Brosius et al., 1981). PCR was performed using a Taqpolymerase (Cinnagen, Iran). The PCR program using following cycling step, initial denaturation at 96°C for 3 min, and then 25 cycles with denaturation at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min. A final extension was performed at 72°C for 3 min.

The PCR product was purified through 1.0 % agarose gel using the GenepHlowTM Gel/ PCR Kit (Geneaid). DNA sequencing of the purified PCR products was carried out on an ABI Prism® 3730XL DNA Sequence (Applied Biosystems, Foster City, California, USA). The sequences were aligned using program CLUSTAL X (version 1.8) (Thompson, 1997) in BioEdit program (Hall, 1999). Phylogenetic trees were reconstructed by the neighbor-joining method (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1985) tree-making algorithms by using the MEGA version 6.0 (Tamura et al., 2013).

Optimisation of *Bacillus* sp. SNRUSA4 Growth Conditions for PLA-food-packaging Degradation using Statistical Design

The optimisation of *Bacillus* sp. SNRUSA4 growth conditions for enhancement of degradation of PLA-food-packaging was investigated. RSM based on BBD was used to design and optimise the culture condition of *Bacillus* sp. SNRUSA4 by Design-Expert® Software Version 10 (Trial Version). *YE* concentration, pH_{ini}, *T* and *AS* were optimised in this research. The suitable ranges of each factor for RSM were selected based on the preliminary single factor experiments. The ranges of four independent variables including *YE* concentration (0.2-3.0 %), pH_{ini} (4.0-10.0), *T* (27-47°C) and *AS* (150-250 rpm) are shown in Table 1. The OD of *Bacillus* sp. SNRUSA4 was examined after 2 days of incubation.

Table 1
Coded and levels of factors in BBD

Independent variables	Code	Levels		
		-1	0	1
YE concentration (%)	X_1	0.2	1.6	3.0
pH_{ini}	X_2	4.0	7.0	10.0
T (C)	X_3	27	37	47
AS (rpm)	X_4	150	200	250

The results of optimisation were confirmed by OD after 2 days of cultivation and degradation of PLA-food-packaging after 4 weeks of cultivation under the optimal conditions based on the results of RSM.

RESULTS AND DISCUSSION

Screening of PLA-food-packaging-degrading Bacteria

Only one bacterial strain, SNRUSA4 revealed increasing OD at 0.203 in BM with PLA-food-packaging as sole carbon source after 4 weeks of cultivation (Figure 1). In contrast, the OD of all controls (the sterile BM, the sterile BM containing 1 g of PLA-food-packaging coupons and the sterile BM with SNRUSA4) did not increase after 4 weeks of cultivation (Figure 1). SNRUSA4 could grow in the BM medium with PLA-food-packaging coupons

which indicated that it was able to degrade the PLA-food-packaging material as a carbon source. The increase of cell density in BM medium supplemented with a bioplastic as a carbon source could indicate the capability of microbe to degrade bioplastic (Jeon & Kim, 2013; Lee et al., 2013; Yottakot & Leelavatcharamas, 2019).

The bacterial strain SNRUSA4 was isolated from compost. This result is also consistent with the study of Kim & Park (2010) and Jeon & Kim (2013). Kim & Park (2010) could isolate PLA-degrading bacteria, *Bordetella petrii* PLA-3 from compost. Jeon & Kim (2013) found *Stenotrophomonas maltophilia* LB 2-3 from compost which could degrade PLA. Therefore, compost is a suitable bacterial source sample to isolate and screening the PLA-degrading bacteria.

The PLA-food-packaging coupons had a weight loss of 7.30±0.11% after degradation by SNRUSA4 for 4 weeks. The weight loss of PLA-food-packaging coupons did not decrease in the absence of bacterial cells. The weight loss can be applied to measure degradation of the plastics (Muhonja et al., 2018). Although, the SNRUSA4 strain can degrade the PLA-food-packaging coupon, the PLA-food-packaging degradation of this strain seems to be very low. The increasing nutrients such as *YE* and gelatin in BM medium can lead to an increase in PLA degradability of microorganism (Jarerat & Tokiwa, 2003; Jarerat et al., 2003; Konkit et al., 2012; Zhou et al., 2017; Bubpachat et al., 2018; Yottakot & Leelavatcharamas, 2019; Decorosi et al., 2019). Thus, the optimisation of culture conditions is essential for the degradation of PLA-food-packaging.

The scanning electron micrographs of the PLA-food-packaging coupons before and after 4 weeks of degradation are shown in Figure 2. There was no significant difference between the surface of PLA-food-packaging coupons before degradation (Figure 2a) and

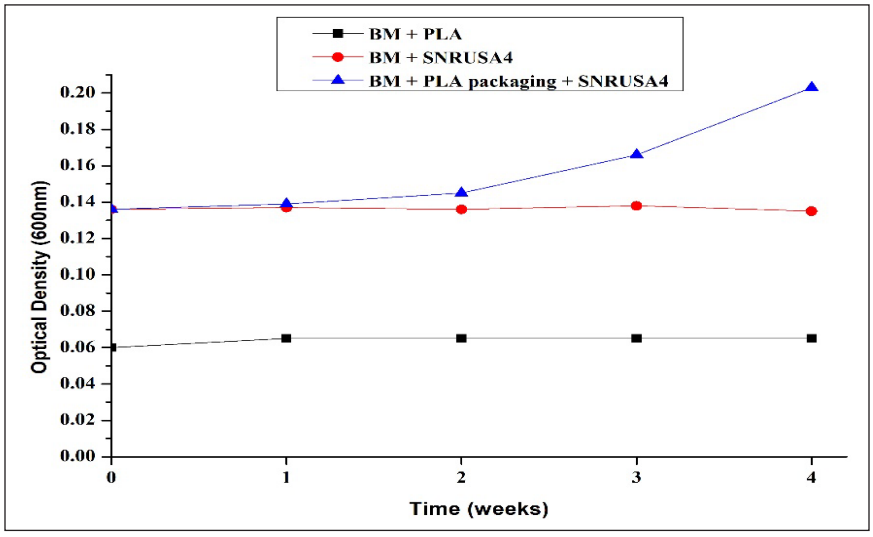
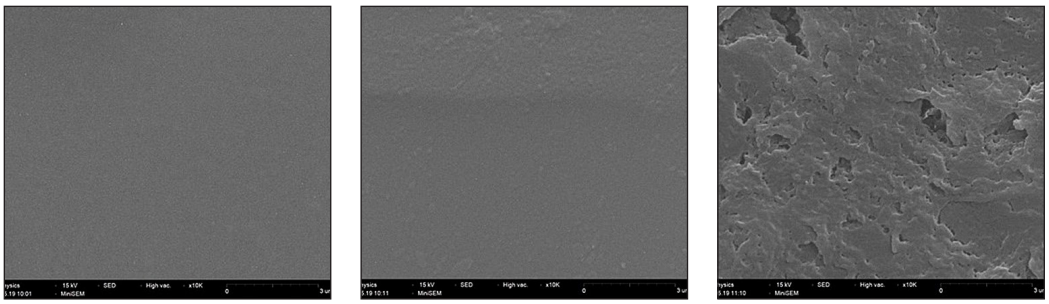


Figure 1. The increase in cell density of strain SNRUSA4 after incubated in the sterile BM medium containing PLA-food-packaging for 4 weeks

the surface of PLA-food-packaging coupons after degradation in BM medium without inoculation of SNRUSA4 (Figure 2b). Figure 2c showed the roughness and porosity on the surface of the PLA-food-packaging coupons after degradation in BM medium with inoculation of SNRUSA4. This finding is similar to the result of Kim et al. (2017) evaluating the morphological changes in the surface of PLA film after the degradation by using SEM.

The standard clear zone method was commonly used to analyse the PLA degradation by microorganism (Liang et al., 2016; Bubpachat et al., 2018; Butbunchu & Pathom-Aree, 2019). The clear zone formation around the colony was formed by microbe able to degrade suspended PLA in emulsified PLA agar medium. This method is therefore applicable for the confirmation of PLA degradation by PLA-food-packaging-degrading bacterial strain SNRUSA4. SNRUSA4 could degrade PLA in emulsified PLA agar plate due to the clear zone formation around the colony within 2 weeks of incubation at 37°C (Figure 3). The microorganisms can produce enzymes to degrade the biopolymers (Penkhrue et al., 2015;



(a) (b) (d)

Figure 2. SEM micrographs of the surface of PLA-food-packaging coupons: (a) the surface before degradation; (b) the surface after incubation in BM medium without the strain; and (c) after the SNRUSA4 strain degradation

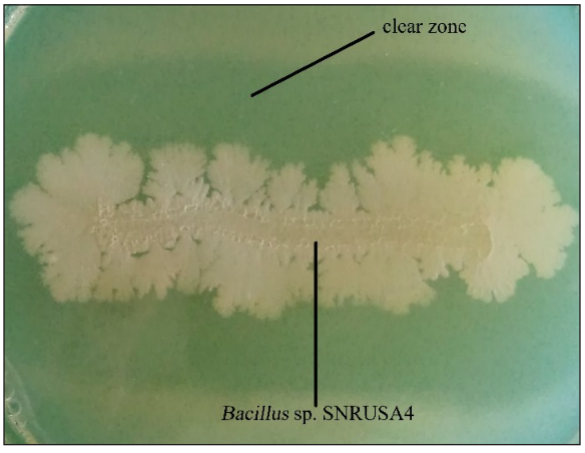


Figure 3. Clear zone formation by *Bacillus* sp. SNRUSA4 on emulsified PLA agar after 2 weeks of incubation at 37°C

Phukon et al., 2012). The formation of clear zone around SNRUSA4 colonies indicates that SNRUSA4 can use PLA as sole carbon source for growth.

Identification of SNRUSA4 Strain

The 16S rDNA sequence of the SNRUSA4 strain is related to more than 99% with the genus *Bacillus*. This isolate is named as *Bacillus* sp. SNRUSA4 (Figure 4). The 16S rDNA sequence of *Bacillus* sp. SNRUSA4 showed similarity with *Bacillus methylotrophicus* KACC 13105 (99.93%), *Bacillus siamensis* KCTC 13613 (99.85%), *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 (99.78%), *Bacillus subtilis* subsp. *subtilis* NCIB 3610 (99.70%), *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7 (99.63%), *Bacillus subtilis* subsp. *inaquosorum* KCTC 13429 (99.63%), *Bacillus tequilensis* KCTC 13622 (99.63%), *Bacillus vallismortis* DV1-F-3 (99.55%), *Bacillus atrophaeus* JCM 9070 (99.48%), *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049 (99.48%), *Brevibacterium halotolerans* DSM 8802 (99.48%), *Bacillus mojavensis* RO-H-1 (99.41%) and *Bacillus vanillea* XY18 (99.40%). The 16S rDNA sequence of *Bacillus* sp. SNRUSA4 was submitted in the GenBank database

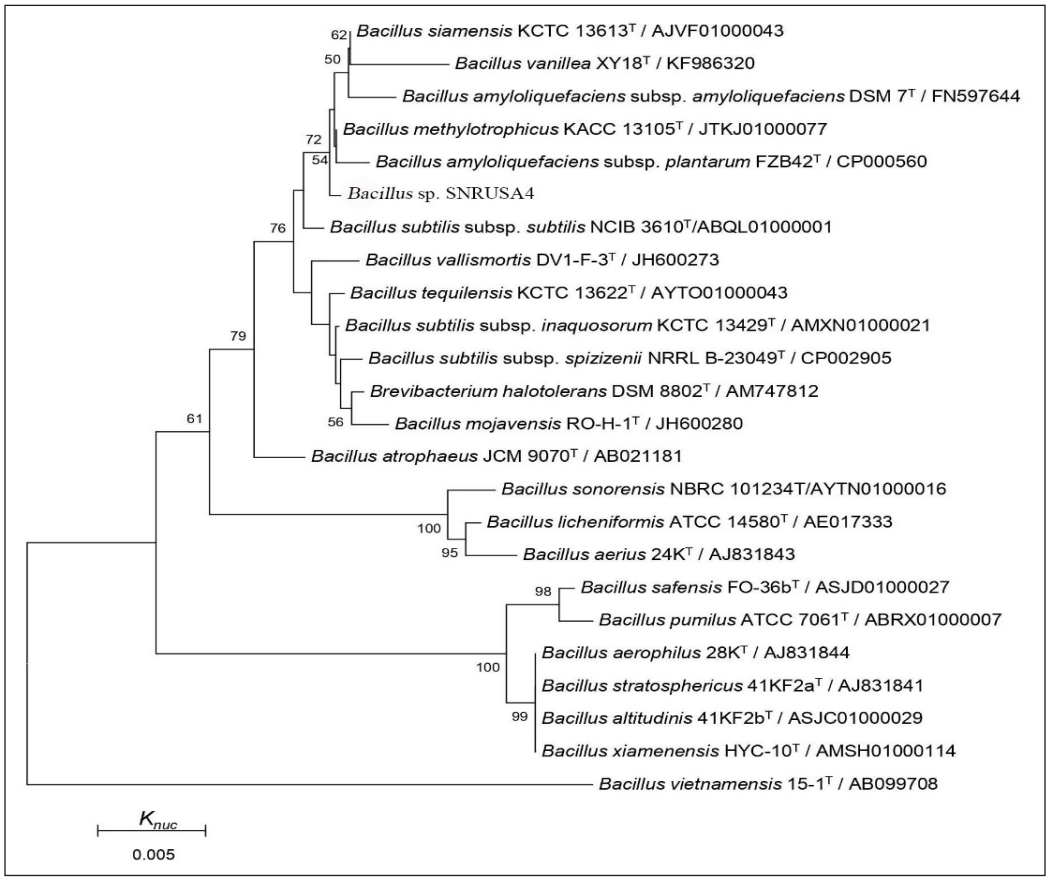


Figure 4. Phylogenetic tree of strain SNRUSA4 and related species of the genus *Bacillus*

system with the accession number MT913031. All of thirteen *Bacillus* species have not been reported yet to being able to degrade PLA. Therefore, *Bacillus* sp. SNRUSA4 is a novel species for PLA and PLA-food-packaging degradation.

Optimization of Growth Conditions by *Bacillus* sp. SNRUSA4

The four factors including *YE* concentration (%) (X_1), pH_{ini} (X_2), *T* (°C) (X_3) and *AS* (rpm) (X_4) were optimised by RSM using BBD. Twenty-eight experiments with three levels of each factor and its responses are shown in Table 2.

Table 2
Experimental conditions of Box Behnken design for Bacillus *sp. SNRUSA4 growth*

Run No.	*YE* concentration (%)	pH_{ini}	*T* (°C)	*AS* (rpm)	Optical Density (600nm)	
					Experiment	Predicted
1	3.0	7	37	250	1.912	1.687
2	0.2	4	37	200	0.007	-0.247
3	1.6	10	27	200	0.034	0.125
4	1.6	10	37	150	0.079	0.073
5	1.6	7	37	200	1.849	1.787
6	3.0	7	37	150	1.666	1.501
7	0.2	7	47	200	0.711	1.029
8	1.6	7	47	150	0.740	0.888
9	1.6	4	47	200	0.009	-0.268
10	1.6	7	27	150	1.545	1.503
11	0.3	10	37	200	0.029	0.242
12	0.2	7	37	250	1.250	1.228
13	1.6	10	47	200	0.011	-0.255
14	1.6	4	37	250	0.009	0.241
15	1.6	7	47	250	1.324	1.325
16	0.2	10	37	200	0.003	-0.232
17	1.6	7	27	250	1.638	1.449
18	3.0	4	37	200	0.014	0.208
19	1.6	7	37	200	1.743	1.787
20	3.0	7	27	200	1.953	1.863
21	0.2	7	27	200	0.621	0.772
22	1.6	4	27	200	0.010	0.900
23	0.2	7	37	150	0.991	1.030
24	1.6	7	37	200	1.819	1.787
25	3.0	7	47	200	0.794	0.868
26	1.6	10	37	250	0.051	0.253
27	1.6	7	37	200	1.738	1.787
28	1.6	4	37	150	0.014	0.037

The results of the experimentation obtained from BBD were fitted to a second order polynomial model to explain the dependence of *Bacillus* sp. SNRUSA4 growth on the four factors. The response surface regression model is shown as Equation 2:

$$Y = -13.47006 + (1.47093X_1) + (2.35354X_2) + (0.23731X_3) + (0.014561X_4) + (0.00113095X_1X_2) - (0.022357X_1X_3) - (0.0000464286X_1X_4) - (0.000183333X_2X_3) - (0.000038333X_2X_4) + (0.0002455X_3X_4) - (0.14887X_1^2) - (0.16692X_2^2) - (0.00361917X_3^2) - (0.00005.34667X_4^2) \quad [2]$$

where Y is the predicted OD, and X_1, X_2, X_3 and X_4 are the independent variables of *YE* concentration, pH_{ini}, T and *AS*, respectively.

The significance and adequacy of the quadratic response surface model was tested by analysis of variance (ANOVA). The results of the second order response surface model fitting in the form of ANOVA are given in Table 3. Even though, the lack of fit was significant (0.0120), the response surface regression model was highly significant with P-value <0.0001 and F-value = 19.33. In addition, the coefficient of determination R^2 (0.9542) and the adjusted coefficient of determination R^2 (0.9048) were also found acceptable (> 0.90) (Chen et al., 2009), which indicated the suitability of the response surface regression model for accurate prediction and analysis of *Bacillus* sp. SNRUSA4 growth.

The linear effect of *YE* concentration and *T* showed significant impact on OD of *Bacillus* sp. SNRUSA4 with P-value of 0.0052 and 0.0194, respectively. The quadratic effect of *YE* concentration (p-value = 0.0106), pH_{ini} (p-value <0.0001) and *T* (p-value = 0.0027) presented great influence on the cell density, indicating that the effect of various factors on OD of *Bacillus* sp. SNRUSA4 was not a simple linear relationship but a significant surface relationship (Yun et al., 2018). There is an interaction between *YE* concentration and *T* because the interaction effect between two factors was significant.

RSM was used to predict the optimal culture conditions of *Bacillus* sp. SNRUSA4. The three-dimensional (3D) response surface plots were generated from regression equation by considering two test variables at one time, while the other two variables were maintained at their middle level. The relationship between the variables and response was visualised through 3D response surface or contour plot to analyse the effects of each factors on the growth of *Bacillus* sp. SNRUSA4. The optimal level of each factor for maximal response, the impact of independent factors and the impact of interaction effect of each factor were determined with the aid of 3D response surface plots. The 3D response surface plots and contour plots are demonstrated in Figure 5(A-F). The shape of 3D response surface plots was convex, which indicated that the well-defined optimal variables were found.

Table 3
Analysis of variance (ANOVA) for BBD results used for optimizing growth by Bacillus *sp. SNRUSA4*

Source	SS	df	MS	*F*-value	*P*-value
Model	15.56	14	1.11	19.33	<0.0001
YE concentration	0.65	1	0.65	11.27	0.0052
pH_{ini}	0.001728	1	0.001728	0.030	0.8650
T	0.41	1	0.41	1.91	0.0194
AS	0.11	1	0.11	0.280	0.1899
YE concentration *pH_{ini}	0.00009025	1	0.00009025	0.00157	0.9690
YE concentration**T*	0.39	1	0.39	6.82	0.0216
YE concentration **AS*	0.00004225	1	0.00004225	0.0007348	0.9788
pH_{ini}**T*	0.000121	1	0.000121	0.002105	0.9641
pH_{ini}**AS*	0.0001323	1	0.0001323	0.0023	0.9625
*T***AS*	0.060	1	0.060	1.05	0.3246
YE concentration **YE* concentration	0.51	1	0.51	8.89	0.0106
pH_{ini} * pH_{ini}	13.54	1	13.54	235.52	<0.0001
T * *T*	0.79	1	0.79	13.67	0.0027
AS * *AS*	0.11	1	0.11	1.86	0.1953
Lack of Fit	0.74	10	0.074	24.06	0.0120
Pure Error	0.009205	3	0.003068		
Cor Total	16.30	27			
$R^2 = 0.9542$ Adjusted $R^2 = 0.9048$ Predicted $R^2 = 0.7382$					

YE is one of the important factors for the optimisation of the growth conditions. It is a complex organic nitrogen source not only contain nitrogen but also certain vitamin B, sulphur, and trace nutrients, and widely used as an ingredient in the media for microorganism cultivation (Kalil et al., 2008). As shown in Figure 5(A), (B) and (C), when we increased the concentration of *YE* up to 2.56%, a maximal growth of *Bacillus* sp. SNRUSA4 was observed. The results of this study indicate that *YE* is a necessary factor for *Bacillus* growth and are similar to other *Bacillus* spp. such as *Bacillus licheniformis* (Hassaan et al., 2014), *Bacillus subtilis* SF4-3 (Tian et al., 2016), *Bacillus* sp. (Biniarz et al., 2018) and *Bacillus aryabhattai* KIIT BE-1 (Ojha et al., 2020).

The pH_{ini} of culture medium plays significant role in bacterial growth. It was clear from Fig. 5(B), (D) and (E) that the medium with pH 7.02 was found to be the best pH_{ini} providing the highest *Bacillus* sp. SNRUSA4 growth. The optimal pH of most *Bacillus* was discovered at neutral pH such as *Bacillus megaterium* (Mohanrasu et al., 2020), *Bacillus cereus* sp. BNPI-92 (Mohammed et al., 2019) and *Bacillus subtilis* (Kim et al., 2020).

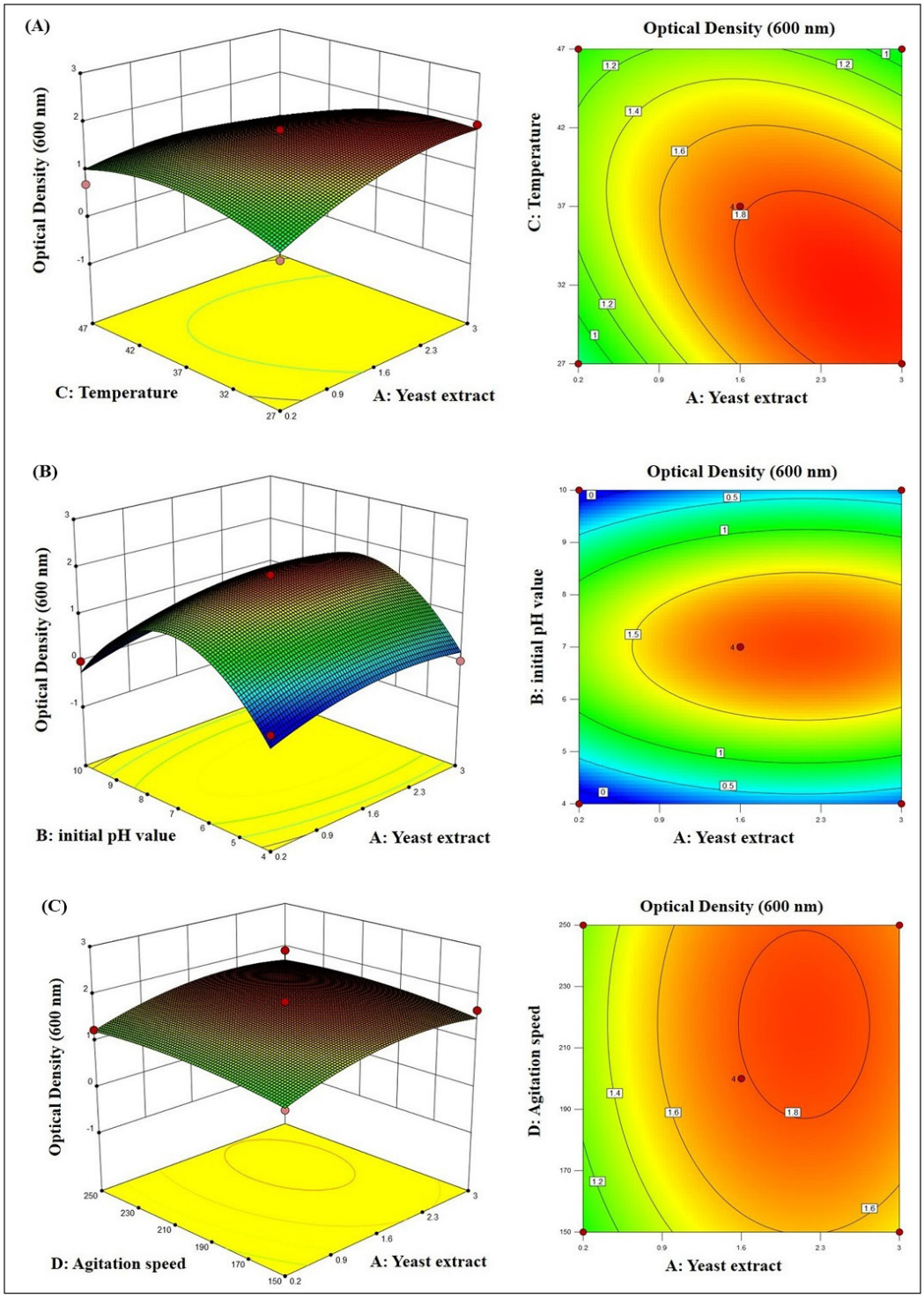


Figure 5. The 3D plots and contour plots showing the effect of (A) *YE* concentration and *T*, (B) *YE* concentration and pH_{ini}, and (C) *YE* concentration and *AS*

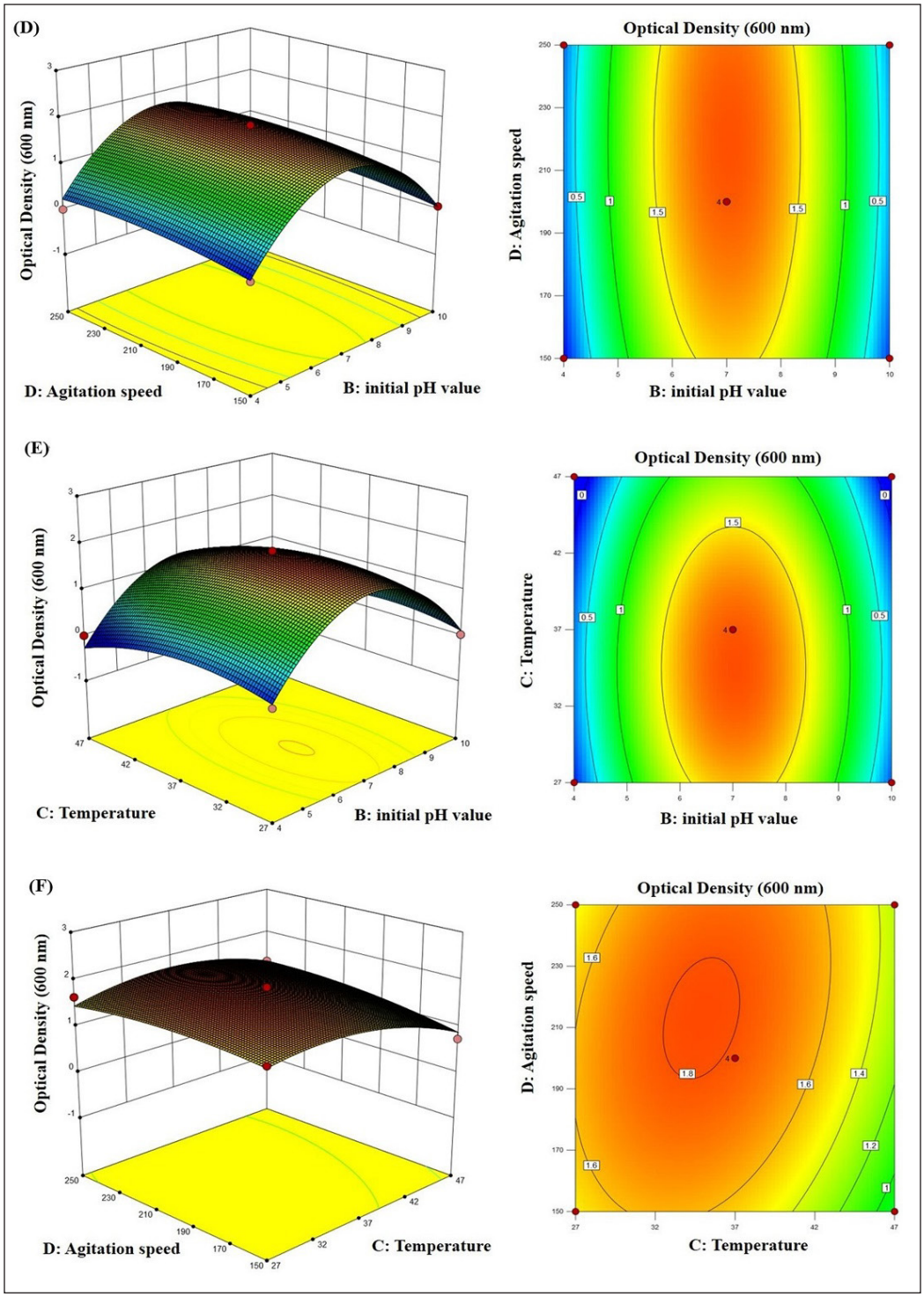


Figure 5. (continue) The 3D plots and contour plots showing the effect of (D) pH_{ini} and *AS*, (E) pH_{ini} and *T*, (F) *T* and *AS*.

T is an important environmental variable in microbial culture influencing enzyme activity, metabolite production and microbial growth (Logan & Vos, 2015). The optimal *T* of *Bacillus* sp. SNRUSA4 from the response surface analysis was 31.68°C [Figures 5(A), (E) and (F)]. *Bacillus* spp. are mesophilic bacteria that grow best at optimal *T* range of 30-37°C. (Khatoon & Rai, 2020; Naskar et al., 2020; Kim et al., 2020).

The *AS* directly affects the oxygen transfer rate (OTR), a parameter that plays an important role in metabolism of aerobic *Bacillus* spp. (Bratcher, 2018). The *AS* of 205.28 rpm was optimal *AS* for *Bacillus* sp. SNRUSA4 cultivation [Figure 5(C), (D) and (F)]. This result conforms with a previous report by Unrean et al. (2012) who found that the highest cell density of *Bacillus subtilis* K-C3 was achieved at *AS* of 200 rpm.

In conclusion, the predicted optimal conditions of *YE* concentration, pH_{ini}, *T* and *AS* from the response surface curves and contour plots were 2.56%, 7.02, 31.68°C and 205.28 rpm, respectively. At these conditions, predicted OD of *Bacillus* sp. SNRUSA4 was 1.921 (Table 4).

Table 4
The optimal values of test factors, and predicted maximum optical density

Variables	Value
YE concentration (%)	2.56
pH_{ini}	7.02
T (°C)	31.68
AS (rpm)	205.28
predicted maximum optical density	1.921

The verification experiment was carried out under the optimal conditions based on the results of RSM for the model validation. The OD after 2 days of cultivation and PLA-food-packaging degradation after 4 weeks of cultivation were investigated to confirm the accurate optimisation results. The predicted OD (1.921) was close to the OD of *Bacillus* sp. SNRUSA4 at optimal conditions (1.955). The result of this experiment proved that the model is reasonable. Moreover, regarding the PLA-food-packaging degradation, the weight loss of PLA-food-packaging under the optimal conditions by *Bacillus* sp. SNRUSA4 had greatly increased, up to 87.10±0.16%.

CONCLUSION

Bacillus sp. SNRUSA4 was discovered in this study as a novel PLA-food-packaging-degrading bacteria that could degrade both PLA and PLA-food-packaging. The optimal conditions from RSM based on BBD were *YE* concentration of 2.56%, pH 7.02, incubation *T* of 31.68°C and *AS* of 205.28 rpm. Under these conditions, the OD was found at 1.955, which was 1.752 more than that before optimisation (0.203). Furthermore, the degradation

of PLA-food-packaging under optimal conditions was also higher than before optimisation due to the increasing weight loss of PLA-food-packaging. The degradation of PLA-food-packaging under optimal conditions was almost twelve times greater than before the optimisation. Based on the current study, *Bacillus* sp. SNRUSA4 is a promising strain for the degradation of PLA-food-packaging. The degradation of PLA-food-packaging by *Bacillus* sp. SNRUSA4 in its natural state should be further studied.

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