Effect of Vanillin over the growth and metabolism of *Zymomonas mobilis*

By

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Title: Effect of Vanillin over the growth and metabolism of
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(Asst.Prof.Dr. Tatsaporn Todhanakasem)

Advisor's Signature

Acknowledgement

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Abstract

The objective of the project is to study the potential of toxic resistance in *Zymomonas mobilis* during the fermentation process in order to convert glucose into ethanol as a byproduct. From Lignocellulosic pretreatment, during the process, it develops many kind of toxic compound including Vanillin. Vanillin, the toxic compound, has been used as the toxin for testing the *Z.mobilis*. We study on the effect of Vanillin on the growth of *Z.mobilis* based on CFU, μ (specific growth rate) and rate of sugar consumption in 4 different concentration rate of Vanilin in *Z.mobilis*; 0 ppm, 50 ppm, 100 ppm, 250 ppm.At concentration of Vanillin 250 ppm showed the significant effect over the growth of *Z.mobilis* by $P<0.03$ on CFU and $P<0.0588$ of specific growth rate. In addition, metabolic of *Z.mobilis* was significant effected when 250 ppm of Vanillin was added.

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Introduction

Lignocellulosematerials have been widely abandoned in the world and there have been considered as the production of biofuel and bioethanol. The structure of lignocellulose is composed of polymers that are cellulose, hemicellulose and an aromatic polymer or lignin. The carbohydrate polymers inside the lignocellulose contain six and five carbon sugars and they are tightly bound to lignin. Lignocellulosicmaterial were classified into virgin biomass, waste biomass and energy crops. Virgin biomass includes all naturally occurring terrestrial plants such as trees, bushes and grass. Waste biomass is produced as a low value byproduct of various industrial sectors such as agricultural waste (corn, sugarcane bagasse, straw etc.), forestry (saw mill and paper mill discards). Energy crops are crops with high yield of lignocellulosic biomass produced to serve as a raw material for production of second generation biofuel examples include switch grassand Elephant grass.

During the process of lignocellulosic pretreatment which is the process for liberating the cellulose from the lignin seal and its crystalline structure through either physical or chemical pretreatments, there are many kind of toxic compounds were produced as the fermentation inhibitors. These inhibitors that were produced from the pretreatment process were capable to inhibit the bioethanol production efficiency bysubsequent hydrolysis and fermentation processes and alsoincrease the cost of production due to entailed detoxification steps.

Vanillin is a phenolic aldehyde, which is an organic compound with the molecular formula $C_8H_8O_3$. Its functional group includes aldehyde, hydroxyl, and ether. It was synthesized from lignin-containing "brown liquor", a byproduct of the lignocellulosic pretreatment process. Vanillin is the capable inhibitor that can inhibit the Bio-ethanol efficiency by affecting the growth rate of microorganisms during the fermentation process and also the interpreting metabolic efficiency or microbe itself.

The concentration rate of vanillin was studied to indicate the specific concentration of vanillin that significantly affect on ethanol production efficiency in long term.

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Objectives

1. To study the effect of various vanillin concentrations based on CFU/ml and specific growth of *Z. mobilis*.
2. To study the effect of vanillin at various concentration on metabolic activity of *Z. mobilis*.

Literature Review

1. *Zymomonas mobilis*

Z. mobilis is the gram-negative rod shaped bacteria. The *Z. mobilis* can be easily found in many origin likes the plant with high percentage of carbohydrate or related crops. The length of the bacteria is 3-6 μm, width is 1- 2 μm. the specific range of proper temperature for growing of the bacteria is 30 – 33 ^{O}C.

Figure 1: The influence of centrifugation on *Z. mobilis* aggregation

(http://www.ejbiotechnology.info/content/vol5/issue3/full/7/f5.html)

In the ethanol fermentation, *Z. mobilis*convertsglucose to pyruvate by using the Entner-Doudoroff pathway. The pyruvate is fermented to produce ethanol and carbon dioxide as the only products.
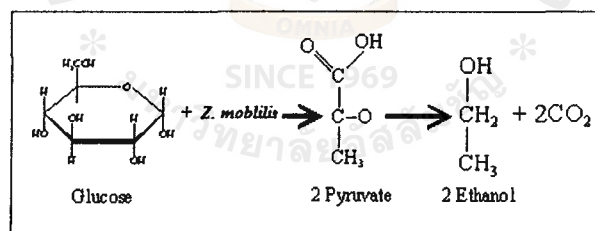
Figure 2: Ethanol fermentation pathway by using *Z. mobilis*

(http://dwb4.unl.edu/Chem/CHEM869P/CHEM869PLinks/www-dept.usm.edu/~bsclabs/380/yeasts.htm)

There weremany advantages of *Z. mobilis* over *S. cerevisiae* with respect to producing bioethanol such as *Z. mobilis*has higher sugar uptake and ethanol yield if compare with *S. cerevisiae*. *Z. mobilis*can produce lower biomass production with higher ethanol tolerance up to 16% (v/v) and it does not require controlled addition of oxygen during the fermentation and amenability to genetic manipulations.

However, in spite of the advantages, there are several factors prevent the usage of *Z. mobilis* in cellulosic ethanol production such as limitation to glucose, fructose and sucrose. Wild-type *Z. mobilis* cannot ferment C5 sugars like xylose and arabinose which are important components of lignocellulosic hydrolysates.

Z. mobilis cannot tolerate toxic inhibitors present in lignocellulosic hydrolysates such as acetic acid and various phenolic compounds (Joy Doran-Peterson., 2008). The maximum concentration of acetic acid that *Z. mobilis* can tolerate with is 1.5% (w/v) (Yun Wang., 2008). In industrial scale, the *Z. mobilis* has shown the alternative of usage in some field of ethanol production because of its ability as a highly potent ethanol producer which can convert sugars to ethanol and carbon dioxide, exhibiting up to 98% but not widely use in other field due to its inability to convert complex carbohydrate polymers like cellulose, hemicellulose, and starch to ethanol and the limitation of toxic tolerance (Shashi Sharma., 1987).

2. Ethanol Fermentation

Ethanol fermentation or alcoholic fermentation is a biotechnological procedure that capable to convert sugars such as glucose, fructose, and sucrose into the cellular energy and the by-product or metabolic wastes are ethanol and carbon dioxide. The fermentation requires anaerobic fermentation for operating the process properly.
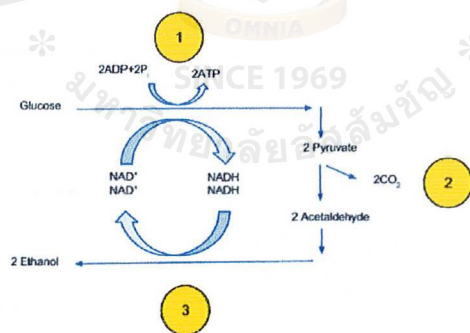
Figure 3: Ethanol fermentation pathway with explanation
(http://en.wikipedia.org/wiki/Ethanol_fermentation)

In ethanol fermentation, glucose was broken down into 2 pyruvates. During the process of break down, the energy from this exothermic reaction is used to bind inorganic phosphates to ADP and convert NAD+ to NADH. Then, 2 pyruvates were broken down into two acetaldehydes and release $2CO_2$. The 2 acetaldehydes were converted to 2 ethanol by using the H- ions from NADH; converting NADH back into NAD.

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There are many raw materials that can be used as the source of sugar in the ethanol fermentation including rice straw, molasses, cassava, corn and many more. due to the structure of the material, the process of fermentation takes time and cost which is limited choice in some countries.

Straw is an agricultural by-product; the dry stalks of cereal plants, after the grain and chaff have been removed. Straw makes up about half of the yield of cereal crops such as barley, oats, rice, rye and wheat. It has many uses, including fuel, livestock bedding and fodder, thatching and basket-making.

Figure 4: Example of rice straw that is used in ethanol production

3. Vanillin

Vanillin is an organic compound. The full name is phenolic aldehyde, the molecular formula $C_8H_8O_3$. The functional groups of vanillin include aldehyde,hydroxyl, and ether.

Figure 5: Vanillin crystals extracted from vanilla extract.

During the pretreatment of lignocellulosic,it produces a wide variety of inhibitory compounds, which strongly inhibit the following enzymatic hydrolysis of cellulosic biomass.

Vanillin is a kind of phenolic derived from degradation of lignin. The effect of vanillin on cellulase activity for the hydrolysis of cellulose was investigated in detail. The results clearly showed that vanillin can reversibly and non-competitively inhibit the cellulase activity at appropriate concentrations and the value of IC50 was estimated to be 30 g/L.
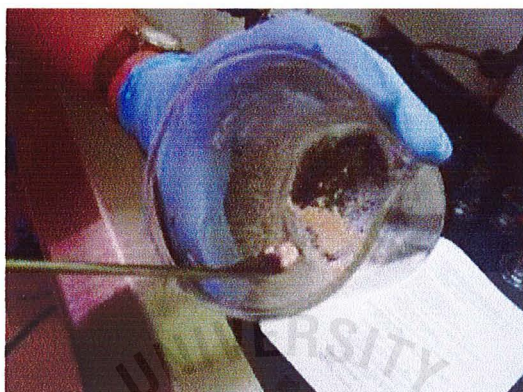
Figure 6: Synthesized vanillin

(http://ochemonline.pbworks.com/w/page/6122881/Vanillin%20Synthesis%20from%204-Hydroxybenzaldehyde)

The inhibition kinetics of cellulase by vanillin was studied using HCH-1 model and inhibition constants were determined. Moreover, investigation of three compounds with similar structure of vanillin on cellulase activity demonstrated that aldehyde group and phenolic hydroxyl groups of vanillin had inhibitory effect on cellulase. These results provide valuable and detailed information for understanding the inhibition of lignin derived phenolic on cellulase (Yun Li., 2014).

Materials and Method

Microorganism

Z. mobilis, strain ZM4 was grown in the yeast peptone glucose (YPG) medium (peptone 10 g, yeast extract 10 g and Glucose 20 g per liter of water) with the pH adjustment is 5.5. The culture was grown at 30 °C to OD_{600} is approximately 1.0.

Preparation of Vanillin

The vanillin was prepared for setting up 4 concentrations of toxic resistance of ZM4 strain which is 0 ppm, 50 ppm, 100 ppm and 250 ppm. The 4 concentrations will be prepared by adding vanillin with distilled water and filtering the solution in each concentration. The concentration of vanillin stock solution was 10 g/L (10,000 ppm) which was used in each concentration later.

Dinitrosalicyclic Acid Reagent Solution

DNS solution or Dinitrosalicyclic Acid Reagent Solution is the solution that is used for testing the reducing sugar in the experimental sample of fermentation process. The DNS test was applied with the sample for checking the level of sugar's reduction which was very effective and useful in term of calculating the consumption rate of microorganism that was studied. The DNS solution contains Dinitrosalicyclic acid 10 g, Phenol 2 g, Sodium sulfite 0.5 g and distills water 1 Liter. (Potassium sodium tartrate solution 40% had been prepared in case of stabilizing the color of the DNS test)

Sugar standard curve

The sugar standard curve was prepared by mix the different rate of glucose with water in the cuvette and using the spectrophotometer to determine the OD_{575} in each rate. After the OD_{575} value in each rate had been collected, the sugar standard curve was plotted between Time and concentration of glucose and determined the R^2 andlinear formula.

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Effect of vanillin on bacterial growth based CFU/ml determination

Vanillin was various added into YPG medium (pH 5.5) at the concentration of 0 ppm, 50 ppm, 100 ppm, 250 ppm. The total 10% v/v of *Z. mobilis* in the 250 ml flask in each concentrationwasdiluted by using serial dilution and the rate of the dilution that has been selected is 10^{-4}. The colony forming unit (CFU/mL) was observed toward 3 days or 72 hours for each vanillin concentration. The data was recorded for finding the difference in each concentration and time.

Maximum specific growth rate (μ_{max}) determination

For analyzing the specific growth rate or μ_{max}, the *Z. mobilis* in vanillin's concentration at 0 ppm, 50 ppm, 100 ppm and 250 ppm were monitored every hour by using spectrophotometry at OD_{600}. The graphs were plotted between OD against time to calculate for maximum specific growth rate (μ_{max}) based on the doubling time (t_d).

Metabolic activity analysis of *Z. mobilis*at various vanillin concentrations

For monitoring the amount of reducing sugar in relevant to the metabolic activity *Z. mobilis*, the culture that had been collected in 3 days in each vanillin concentration werecentrifuged (8,000 rpm for 5 minutes) and collected the supernatant for DNS testing with OD_{575}. The result of OD were collected and calculated based on sugar standard curve for finding the leftover of reducing sugar in each concentration of vanillin

Result & Discussion

Vanillin is the one of the toxic compounds that can be synthesized during the pretreatment process of lignocellulosic waste by acid hydrolysis; many toxic compounds have been reported as toxins that can inhibit the growth of microorganism and the ethanol production in *Z. mobilis*. The concentration of vanillin was varied to study on its effect over the growth and metabolic activity of *Z. mobilis*. For determining the maximum level of vanillin that represents the significant effect over the cell growth and metabolic activity of *Z. mobilis*, vanillin concentration 250 ppm tended to terminate the growth of microorganism at 48 hours while 50 and 100 ppm of vanillin concentration represented only a slight affected over the growth of microorganism based on the analysis of colony forming unit (CFU /ml) (Figure 7).
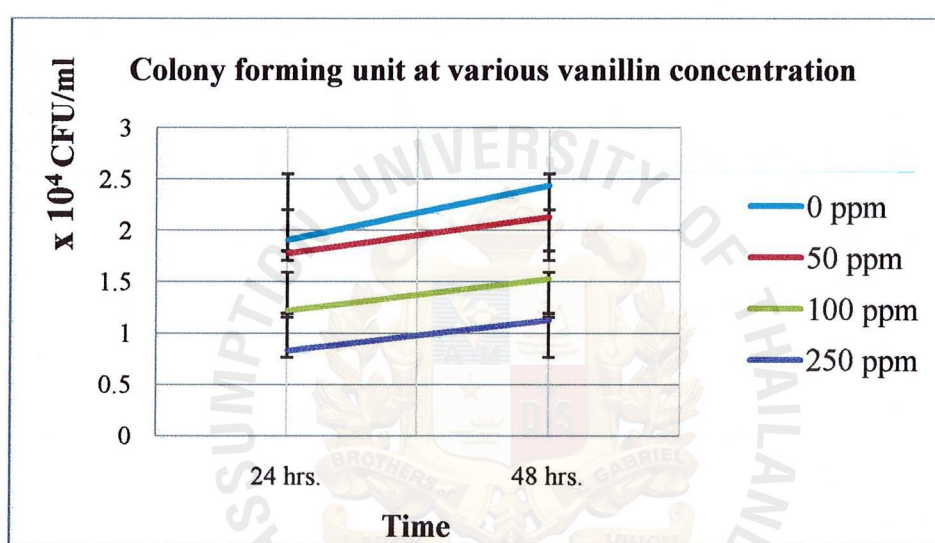
Figure7: The colony forming unit (CFU/ml) in24 and 48 hours of ZM4 under various vanillin concentration (0 ppm, 50 ppm, 100 ppm, 250 ppm)

Table 1: The table illustrated the P – value of colony forming unit (CFU/ml) with various vanillin concentration (0, 50, 100, 250 ppm) at 0, 24 and 48 hours.

Vanillin's concentration	24 hrs.	48 hrs.
0 ppm	1	1
50 ppm	0.7078	0.2945
100 ppm	0.1583	0.0862
250 ppm	0.0712	0.0303[*]

Note: One asterisk represent the $P \leq 0.05$ from two tailed t-test when compared to the control (0 ppm)

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In the maximum specific growth rate (μ_{max}), the growth of *Z.mobilis* was studied by monitoring the specific growth rate in 8 hours for plotting the semi-log curve. During the pretreatment process of lignocellulose, vanillin, represented as byproduct of the process, which had been synthesized from the treatment, this was capable to inhibit the growth of *Z.mobilis* and its metabolism continuously. From the result, the specific growth rate indicated nearly the significant different with the $P \leq 0.05$ at250 ppm of vanillin concentration (table 2).

Table 2: The P- value, $\mu_{Average}$ and standard deviation of specific growth rate of ZM4

Vanillin Concentration	$\mu_{Average} \pm$ S.D.	P - value
0 ppm	1.188 ± 0.280	1
50 ppm	0.661 ± 0.044	0.1196
100 ppm	0.478 ± 0.023	0.0703
250 ppm	0.389 ± 0.061	0.0588*

Note: the one asterisk represent the $P \leq 0.05$ from two tailed t-test when compared to the control (0 ppm)

To determine the amount of glucose that had been used in the metabolism of *Z.mobilis* in order to produce ethanol, the standard sugar curve was plotted for identifying the equation of standard sugar curve which is $x = \dfrac{Y + 0.02}{0.0197}$(Figure 8). The equation was used in DNS test for calculating the amount of sugar that is reduced from the metabolism of ZM4 in the fermentation process.
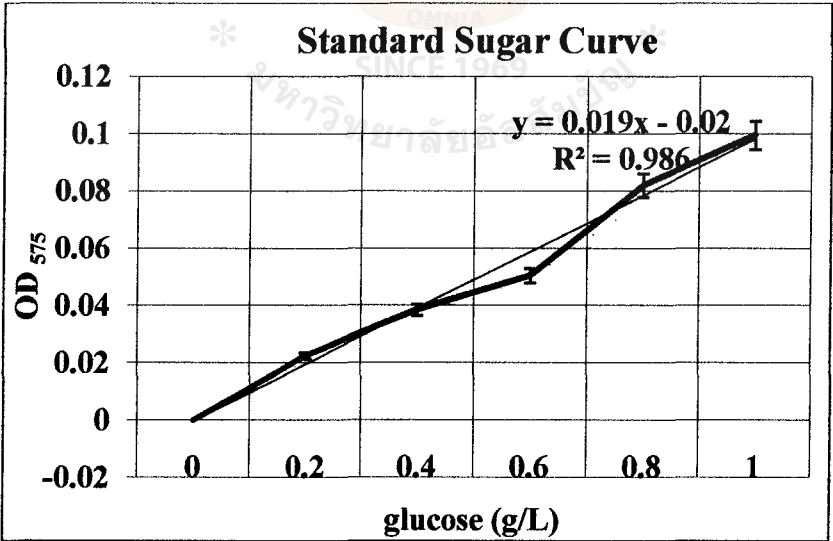

Figure 8: Standard Sugar Curve by using glucose in different concentration of glucose at (g/L) (0, 0.2, 0.4, 0.6, 0.8, 1 g/L) in which analyzed with OD_{575}

In the metabolism of *Z. mobilis*, the glucose is the source of energy that was converted into ethanol under anaerobic fermentation. The amount of glucose was varied due to the effectiveness of microorganism, environmental concentrations and inhibitors that can affect the rate of glucose consumption and ethanol production. In addition, vanillin was the toxic compound that probably affected glucose consumption of *Z. mobilis* directly and cause many problems after. So vanillin was tested on its toxicity based on metabolic activity. One method to determine the sugar concentration of reducing sugars is by heating with 3,5 Dinitrosalicyclic acid(DNS) which produce a red-brown product Miller(1959)The reaction is direct, thus the method is preferred over the Benedict's test method.For the DNS test, the amount of reducing sugar (g/L) has been monitoring by using the linear equation of sugar standard curve for calculating the actual amount of sugar that has been used by the metabolism of ZM4 in 4 different concentration of vanillin (0 ppm, 50 ppm, 100 ppm, 250 ppm) at 0, 24, 48 hours of observation.

As the result, in 0, 24, 48 hours, the amount of reducing sugar in each concentration was decreasing respectively. But the rate of reducing sugar in 250 ppm concentration was clearly different if comparing with others concentration and the time of observation which is 0, 24 hours (Figure 9). The DNS test indicated the significant different with the $P \leq 0.05$ with 250 ppm of vanillin concentration at all-time points start with 0, 24 and 48 hours.(Table 3).
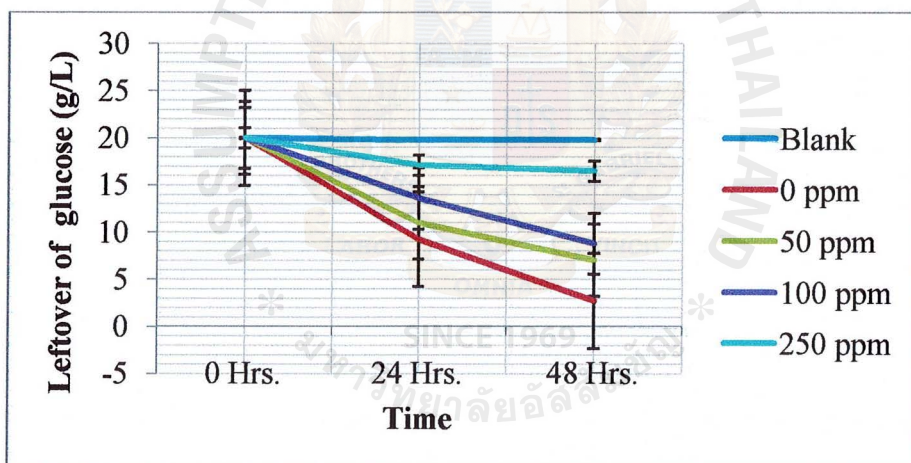

Figure 9: The graph that show the amount of reducing sugar in 4 concentrations of vanillin in different period of time.

Table 3: The table illustrated the P - value with various vanillin concentration (0, 50, 100, 250 ppm) at 0, 24, 48 hours comparing of reducing sugar usage.

P-value	0 hours	24 hours.	48 hours.
YPG + 20 g/L of sugar			
YPG + 0 ppm	-	-	-
YPG +50 ppm	0.612	0.7391	0.0131
YPG +100 ppm	0.0972	0.0859	0.0068*
YPG +250 ppm	0.0316*	0.026*	0.0016*

Note: the one asterisk represent the $P \leq 0.05$ from two tailed t-test when compared to the control (0 ppm)

According to the result of vanillin test for inhibition of *Z. mobilis* (Mary Ann Franden, 2013), the concentration of vanillin that had effect with the growth rate of *Z. mobilis* is 20 mM (300 ppm) which can be the initiated concentration for disturbing the time of growth rate and the ethanol production by *Z. mobilis* respectively.Comparing with other study of vanillin's effect in other lignocellulosic hydrolysates as the inhibitor, vanillin had been identified and studied as the effective inhibitor. The study results of vanillin in the growth of the fermenting yeast, *S. cerevisiae* CEN.PK 113-7D showed thatthe vanillin were capable to prolong lag-phase of the growth of bacteria. (Ying Zha and Johan A Westerhuis, 2014)

Conclusion

Z. mobilis, the ZM4 strain was affected from the vanillin. Vanillin at the concentration rate 250 ppm was capable to inhibit the growth, and presuming on ethanol production of ZM4. It appeared that any concentration above the limitation of 250 ppm was possible to cause the effect of inhibition in the same way. The presence of vanillin, the toxic compound can illustrate the significant effect on both microbial growth and ethanol production respectively. And the detoxification process must be suggested in order to eliminate these compounds to be less 250 ppm to minimize its effect over microbial growth and its metabolic activity.

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Appendix:

Experimental Data

Colony Forming Unit result

Table 4: The table illustrated the colony forming unit (CFU/ml) at various vanillin concentration (0, 50, 100, 250 ppm) at 0 hours.

0 hours.	1st trial	2nd trial	Average± S.D.
0 ppm	N/A	N/A	-
50 ppm	N/A	N/A	-
100 ppm	N/A	N/A	-
250 ppm	N/A	N/A	-

Table 5: The table illustrated the colony forming unit (CFU/ml) at various vanillin concentration (0, 50, 100, 250 ppm) at 24 hours.

24 hours.	1st trial	2nd trial	Average± S.D.
0 ppm	1.63×10^7	2.19×10^7	$1.91 \times 10^7 \pm 3.95 \times 10^6$
50 ppm	1.89×10^7	1.67×10^7	$1.78 \times 10^7 \pm 1.55 \times 10^6$
100 ppm	1.36×10^7	1.08×10^7	$1.22 \times 10^7 \pm 1.97 \times 10^6$
250 ppm	7.1×10^6	9.5×10^6	$8.3 \times 10^7 \pm 1.6 \times 10^6$

Table 6: The table illustrated the colony forming unit (CFU/ml) at various vanillin concentration (0, 50, 100, 250 ppm) at 48 hours.

48 hours.	1st trial	2nd trial	Average ± S.D.
0 ppm	2.27×10^7	2.61×10^7	$2.44 \times 10^7 \pm 2.4 \times 10^6$
50 ppm	2.27×10^7	1.99×10^7	$2.1 \times 10^7 \pm 1.9 \times 10^6$
100 ppm	1.3×10^7	1.76×10^7	$1.53 \times 10^7 \pm 3.25 \times 10^6$
250 ppm	9.7×10^6	1.29×10^7	$1.13 \times 10^7 \pm 2.26 \times 10^6$

Optical Density test

Figure 10: Average Semi- log curve of ZM4 + vanillin conc. 0 ppm in OD_{600} for 8 hours.

Figure 11: Average Semi- log curve of ZM4 + vanillin conc. 50 ppm in OD_{600} for 8 hours.

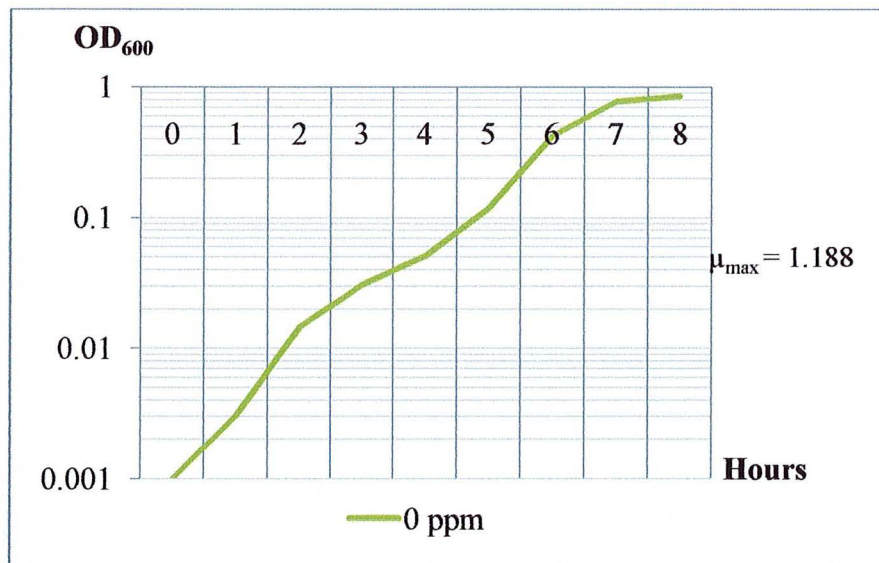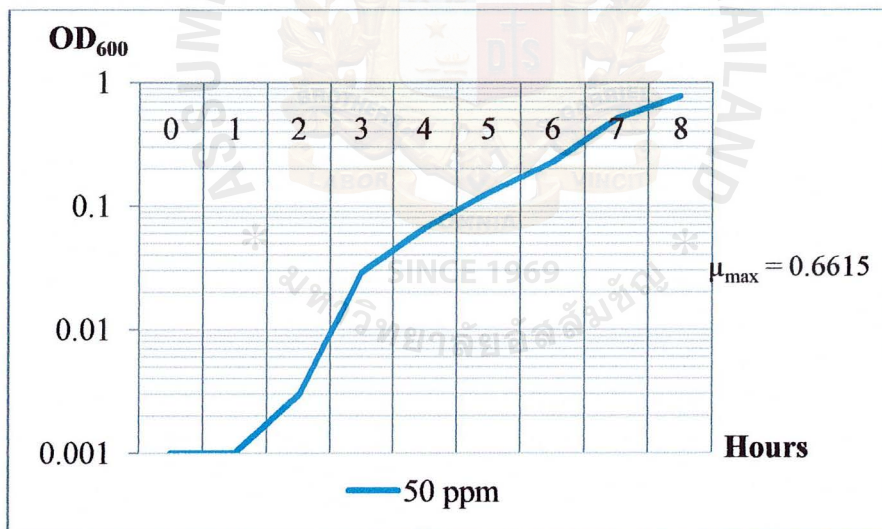Figure 12: Average Semi- log curve of ZM4 + vanillin conc. 100 ppm in OD_{600} for 8 hours.

Figure 13: Average Semi- log curve of ZM4 + vanillin conc. 250 ppm in OD_{600} for 8 hours.

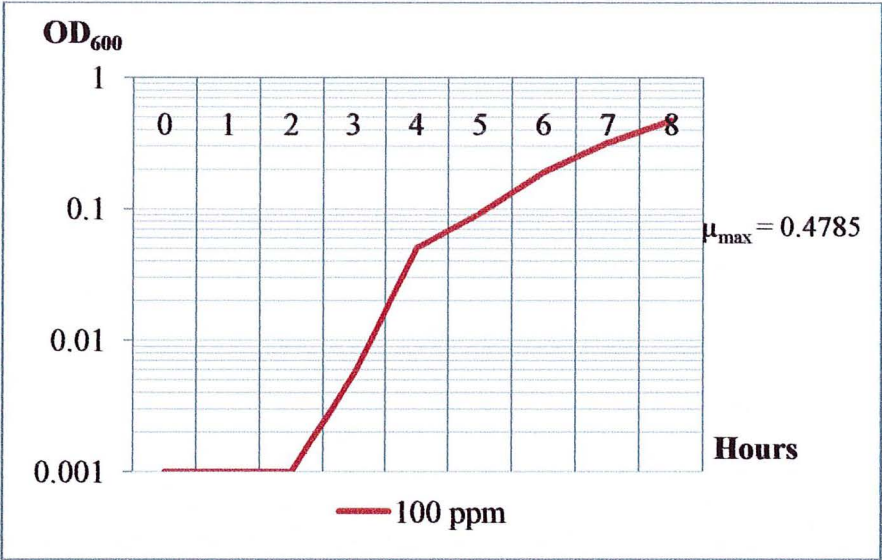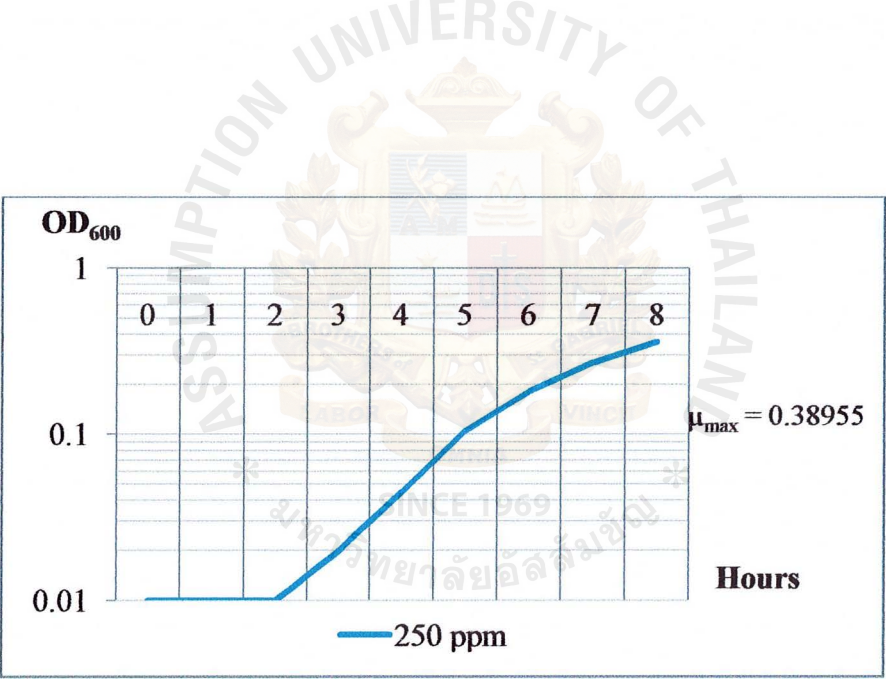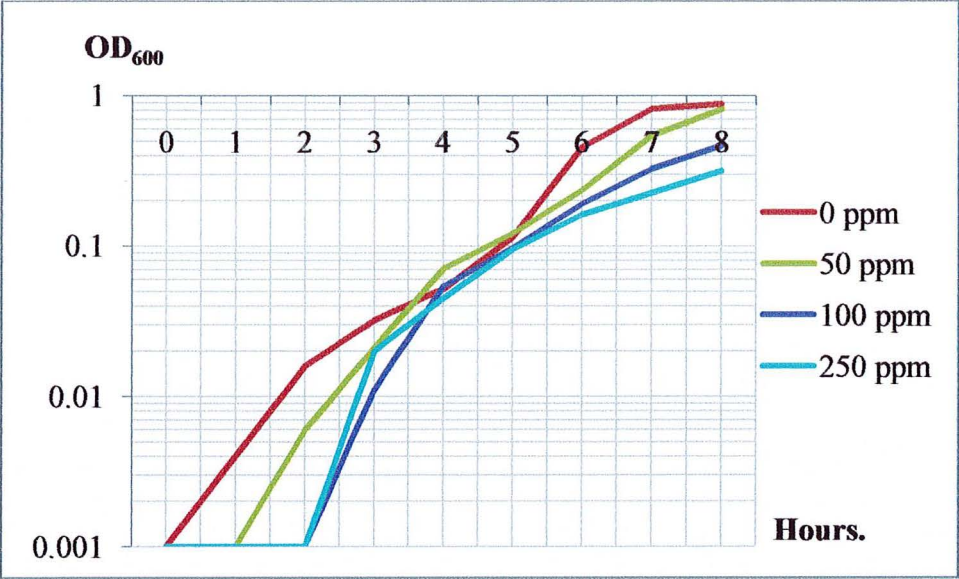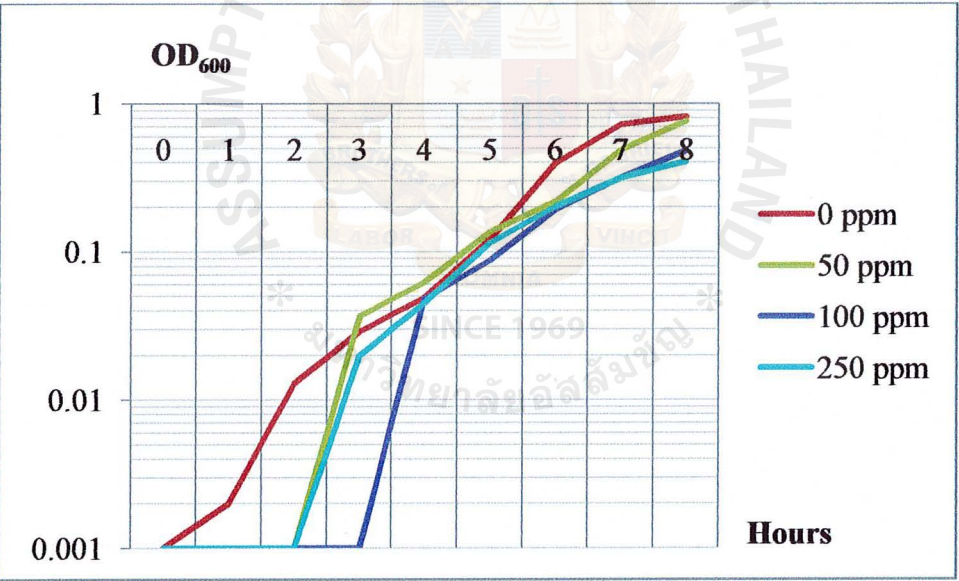Figure 14: 1st trial of Semi-log graph of ZM4 at OD_{575}

Figure 15: 2nd trial of Semi-log graph of ZM4 at OD_{575}
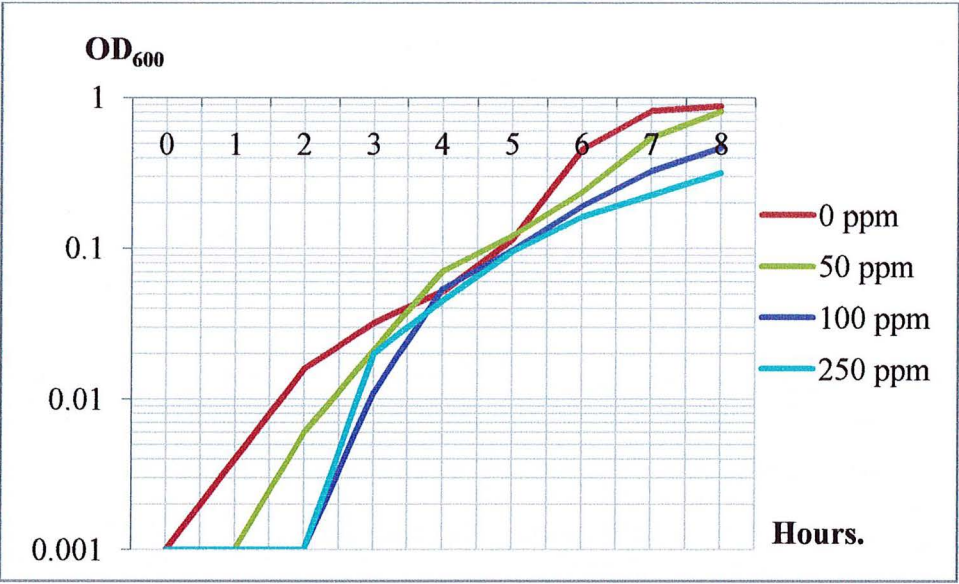
Figure 14: 1st trial of Semi-log graph of ZM4 at OD$_{575}$
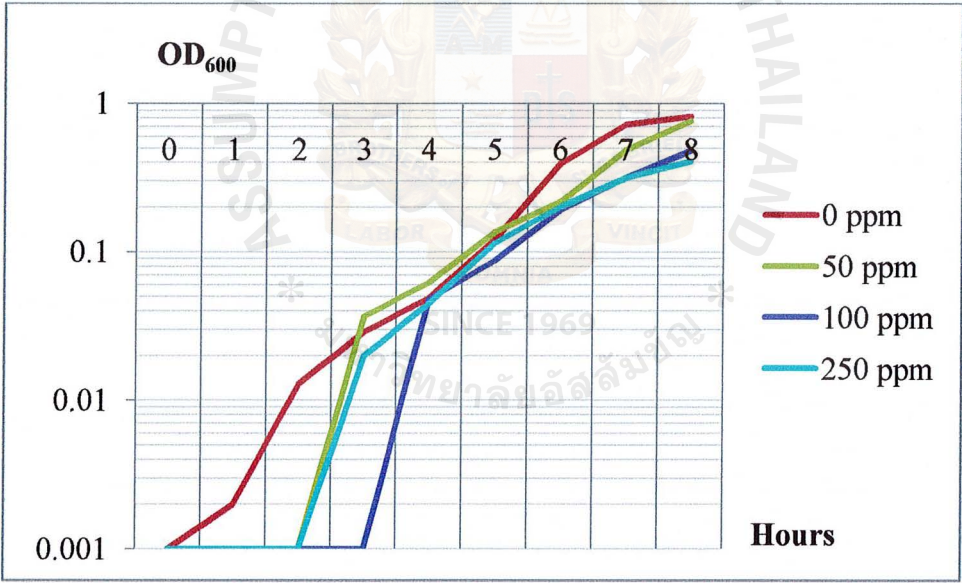
Figure 15: 2nd trial of Semi-log graph of ZM4 at OD$_{575}$

Table 7: the 1st trial of OD test of ZM4 by using wavelength at 600 nm

Hours	0 ppm	50 ppm	100 ppm	250 ppm
0	0	0	0	0
1	0.004	0	0	0
2	0.016	0.006	0	0
3	0.032	0.021	0.011	0.02
4	0.052	0.071	0.054	0.045
5	0.115	0.121	0.098	0.095
6	0.454	0.236	0.191	0.162
7	0.823	0.545	0.327	0.226
8	0.885	0.812	0.467	0.316

Table 8: the 2nd trial of OD test of ZM4 by using wavelength at 600 nm

Hours	0 ppm	50 ppm	100 ppm	250 ppm
0	0	0	0	0
1	0.002	0	0	0
2	0.013	0	0	0
3	0.029	0.037	0	0.02
4	0.049	0.062	0.048	0.045
5	0.121	0.135	0.087	0.115
6	0.394	0.218	0.191	0.202
7	0.723	0.486	0.316	0.315
8	0.821	0.756	0.481	0.406

Table 9: the 1st trial of OD test of doubling time and specific growth rate

Vanillin's concentration	Doubling time (t_d)	Maximum specific growth rate (μ_{max})
0 ppm	0.5	1.386
50 ppm	1.1	0.63
100 ppm	1.4	0.495
250 ppm	1.6	0.4331

Table 10: the 2nd trial of OD test of doubling time and specific growth rate

Vanillin's concentration	Doubling time (t_d)	Maximum specific growth rate (μ_{max})
0 ppm	0.7	0.99
50 ppm	1	0.693
100 ppm	1.5	0.462
250 ppm	1.8	0.346

Standard Sugar Curve data

Table 11: The standard sugar table which is measured by spectrophotometer OD_{575} based on DNS method

g/L	$OD_{1st\ trials}$	$OD_{2nd\ trials}$	$OD_{Average}$ ± Standard deviation
0	0	0	0
0.2	0.018	0.027	0.0225±0.006364
0.4	0.036	0.041	0.0385±0.003536
0.6	0.048	0.053	0.0505±0.003536
0.8	0.073	0.091	0.082±0.012728
1	0.101	0.098	0.0995±0.002121

DNS test data

Table 12: the 1st trial of amount reducing sugar usage in g/L + 1% of DNS at 575 nm

	0 Hrs.	24 Hrs.	48 Hrs.
Blank	0.2	0.2	0.2
0 ppm	1.9	10.74	17.27
50 ppm	1.64	9	12.95
100 ppm	0.95	6.43	11.22
250 ppm	0.22	2.9	3.5

Table 13: the 2nd trial of amount reducing sugar usagein g/L+ 1% of DNS at 575 nm

	0 Hrs.	24 Hrs.	48 Hrs.
Blank	0.2	0.2	0.2
0 ppm	1.4	8.6	16.4
50 ppm	1.3	9.5	12.6
100 ppm	0.8	5.7	10.8
250 ppm	0.3	1.8	4.1

Table 14: the average and standard deviation of amount reducing sugar usage in g/L + 1% of DNS at 575 nm

	0 Hrs.	24 Hrs.	48 Hrs.
Blank	0.2±0	0.2±0	0.2±0
0 ppm	1.65±0.353553	9.67±1.513209	16.835±0.615183
50 ppm	1.47±0.240416	9.25±0.353553	12.775±0.247487
100 ppm	0.875±0.106066	6.065±0.516188	11.01±0.296985
250 ppm	0.26±0.056569	2.35±0.777817	3.8±0.424264

Table 15: The average, standard deviation, P-value (Blank) and P-value (0) of reducing sugar usage in ZM4 at 0 hour.

	Reducing sugar leftover (g/L)in average± Standard Deviation	P-value (blank)	P-value
YPG + 20 g/L of sugar	0		
YPG + 0 ppm	1.65 ± 0.353553391	0.0285	-
YPG +50 ppm	1.47 ± 0.240416306	0.0175	0.612
YPG +100 ppm	0.875 ± 0.106066017	0.0121	0.0972
YPG +250 ppm	0.26 ± 0.056568542	0.2724	0.0316*

Table 16: The average, standard deviation, P-value (Blank) and P-value (0) of reducing sugar usage in ZM4 at 24 hours.

	Reducing sugar leftover(g/L) in average ± Standard Deviation	P-value (blank)	P-value
YPG + 20 g/L of sugar	0		
YPG + 0 ppm	9.67 ± 1.513208512	0.0125	-
YPG +50 ppm	9.25 ± 0.353553391	0.0008	0.7391
YPG +100 ppm	6.065 ± 0.51618795	0.0039	0.0859
YPG +250 ppm	2.35 ± 0.777817459	0.0596	0.026*

Table 17: The average, standard deviation, P-value (Blank) and P-value (0) of reducing sugar usage in ZM4 at 48 hours.

	Reducing sugar leftover(g/L)in average ± Standard Deviation	P-value (blank)	P-value
YPG + 20 g/L of sugar	0		
YPG + 0 ppm	16.835±0.6151829	0.0007	-
YPG +50 ppm	12.775±0.247487373	0.0002	0.0131
YPG +100 ppm	11.01±0.296984848	0.0004	0.0068
YPG +250 ppm	3.5±0.424264069	0.0069	0.0016*

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