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APPENDICES

APPENDIX I

1. Culture media preparation

Corn Meal Agar (CMA)

Ground corn	50 g
Glucose	10 g
Agar	15 g
Distilled water	1 l

Malt Extract Agar (MEA)

Malt extract	25 g
Agar	15 g
Distilled water	1 l

Potato Dextrose Agar (PDA)

Potato (cut, boil & filtered)	200 g
Glucose	20 g
Agar	15 g
Distilled water	1 l

EPS production medium

Glucose	50 g
$(NH_4)_2SO_4$	0.6 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	5.0 g
NaCl	1.0 g
Yeast extract	0.4 g
Distilled water	1 l

Yeast Malt Agar (YMA)

Glucose	20 g
Malt extract	5 g
Yeast extract	5 g
Agar	15 g
Distilled water	1 l

Yeast Malt Broth (YMB)

Glucose	20 g
Malt extract	5 g
Yeast extract	5 g
Distilled water	1 l

10X Yeast Carbon Base (YCB)

Yeast carbon base	6.7 g
Distilled water	100 ml
(Filtered sterile)	

10X Yeast Nitrogen Base (YNB)

Yeast nitrogen base	6.7 g
Distilled water	100 ml
(Filtered sterile)	

2. Chemical preparation

Antrone reagent

Concentrated sulfuric acid	720 ml
Anthrone	500 mg
Thiourea	10 g
Distilled water	280 ml

To 280 ml of distilled water add 720 ml of concentrated sulfuric acid. While this mixture is still warm, add 500 mg of anthrone and 10 g of thiourea and mix until dissolved. Cool and store in the refrigerator. This reagent is most satisfactory if aged at least 4 hours prior to use. It will keep for at least 2 weeks in the refrigerator.

Borohydride-reduced pullulan and borohydride-reduced starch (1% w/v)

Soluble starch (or pullulan)	2.5 g
$NaBH_4$	75 mg
Acetone	0.2 ml
1N acetic acid	
Distilled water	

Gently boil suspension of 2.5 g of soluble starch (or pullulan) in distilled water (10 ml). After suspension cooling down, dilute it up to 45 ml with distilled water. Gradually add cooled solution of $NaBH_4$ 75 mg in distilled water (5 ml) into suspension and magnetically mixing with a Teflon-coat stirrer bar. Incubate suspension in water bath at 25°C over night. Suspension (1 ml) was mixed with 0.2 ml of acetone. Incubate for 20 min at room temperature. Adjust suspension to pH 7.0 using 1N acetic acid. Adjust volume of suspension to 50 ml with assay buffer.

Iodine solution

Iodine stock

KI	1.5 g
Iodine	50 mg

Distilled water

Add KI (1.5 g) and iodine (50 mg) into distilled water. Adjust volume of solution to 25 ml.

Iodine staining solution

Iodine stock	1 ml
KI	5 g

Distilled water

Add iodine stock (1ml) and KI (5 g) into distilled water. After solution mix well, adjust volume to 125 ml.

APPENDIX II

ANOVA and DMRT

Pullulan yield of *Aureobasidium* strain BK4 grown on various conditions

Production period (day)	Production condition/ EPS yield ($g.l^{-1}$)			
	Glucose + $(NH_4)_2SO_4$	Sucrose + $(NH_4)_2SO_4$	Sucrose + $NaNO_3$	Sucrose + Peptone
1	2.4^{m}	2.8^{m}	2.0^{m}	2.1^{m}
2	4.5^{l}	8.4^{ijk}	5.2^{l}	4.6^{l}
3	5.6^{l}	14.1^{efg}	8.8^{ij}	9.2^{i}
4	7.7^{jk}	19.9^{c}	11.7^{h}	13.4^{g}
5	7.9^{jk}	21.3^{b}	12.1^{h}	14.8^{ef}
6	8.0^{jk}	23.1^{a}	14.5^{efg}	18.0^{d}
7	7.5^{k}	19.9^{c}	14.0^{fg}	15.2^{e}

[1]Superscript letters indicate that a value followed by the same letter did not differ significantly ($P<0.05$) in Duncan's multiple range test (DMRT) from other values with the same letter

ANOVA

EPSs from BK4	df	Mean Square	F
Between Groups	27	114.226	260.434*
Within Groups	56	.439	
Total	83		

* = Significant at 95%

Pullulan yield of *Aureobasidium* strain BK6 grown on various conditions

Production period (day)	Production condition/ EPS yield ($g.l^{-1}$)			
	Glucose + $(NH_4)_2SO_4$	Sucrose + $(NH_4)_2SO_4$	Sucrose + $NaNO_3$	Sucrose + Peptone
1	2.1^{lm}	2.0^{lm}	0.3^{m}	1.8^{mn}
2	3.6^{j}	7.5^{g}	5.2^{l}	6.0^{h}
3	4.8^{i}	7.5^{g}	8.8^{ij}	8.0^{h}
4	5.8^{h}	9.1^{f}	11.7^{h}	13.1^{de}
5	5.7^{h}	12.4^{e}	12.1^{h}	13.2^{de}
6	5.9^{h}	13.4^{d}	14.5^{efg}	14.9^{c}
7	6.1^{h}	17.0^{d}	14.0^{fg}	15.9^{b}

[1]Superscript letters indicate that a value followed by the same letter did not differ significantly ($P<0.05$) in Duncan's multiple range test (DMRT) from other values with the same letter

ANOVA

EPSs from BK6	Sum of Squares	df	Mean Square	F
Between Groups	1980.365	27	73.347	279.017*
Within Groups	14.721	56	.263	
Total	1995.086	83		

* = Significant at 95%

Pullulan yield of *Aureobasidium* strain LB3 on various conditions

Production period (day)	Production condition/ EPS yield ($g.l^{-1}$)			
	Glucose + $(NH_4)_2SO_4$	Sucrose + $(NH_4)_2SO_4$	Sucrose + $NaNO_3$	Sucrose + Peptone
1	0.7^{p}	1.9^{no}	1.1^{op}	2.9^{lm}
2	2.2^{mn}	6.1^{i}	2.6^{mn}	6.0^{i}
3	3.6^{kl}	9.9^{fg}	6.6^{i}	8.0^{h}
4	4.1^{k}	10.9^{de}	9.4^{g}	10.6^{def}
5	4.5^{jk}	12.4^{c}	10.2^{efg}	12.1^{c}
6	5.1^{j}	13.8^{b}	11.1^{d}	13.8^{b}
7	4.6^{jk}	12.6^{c}	12.4^{c}	15.2^{a}

[1]Superscript letters indicate that a value followed by the same letter did not differ significantly ($P<0.05$) in Duncan's multiple range test (DMRT) from other values with the same letter

ANOVA

EPSs from LB3	Sum of Squares	df	Mean Square	F
Between Groups	1599.694	27	59.248	198.672*
Within Groups	16.700	56	.298	
Total	1616.394	83		

* = Significant at 95%

Pullulan yield of *Aureobasidium* strain NRM2 on various conditions

Production period (day)	Production condition/ EPS yield ($g.l^{-1}$)			
	Glucose + $(NH_4)_2SO_4$	Sucrose + $(NH_4)_2SO_4$	Sucrose + $NaNO_3$	Sucrose + Peptone
1	1.0^{o}	1.4^{o}	2.3^{no}	2.4^{no}
2	3.8^{mn}	5.6^{jkl}	5.7^{jkl}	6.3^{jk}
3	5.5^{lm}	9.0^{i}	10.9^{h}	12.5^{gh}
4	6.2^{jk}	11.4^{gh}	16.4^{cde}	15.4^{de}
5	6.5^{jk}	13.1^{fg}	16.8^{cd}	18.0^{bc}
6	7.2^{ijk}	14.7^{ef}	17.4^{cd}	24.8^{a}
7	7.7^{ijk}	19.6^{b}	15.8^{de}	25.2^{a}

[1]Superscript letters indicate that a value followed by the same letter did not differ significantly ($P<0.05$) in Duncan's multiple range test (DMRT) from other values with the same letter

ANOVA

EPSs from NRM2	Sum of Squares	df	Mean Square	F
Between Groups	3761.289	27	139.307	104.137*
Within Groups	74.913	56	1.338	
Total	3836.202	83		

* = Significant at 95%

Pullulan yield of *Aureobasidium* strain SK3 on various conditions

Production period (day)	Production condition/ EPS yield ($g.l^{-1}$)			
	Glucose + $(NH_4)_2SO_4$	Sucrose + $(NH_4)_2SO_4$	Sucrose + $NaNO_3$	Sucrose + Peptone
1	0.5^{o}	0.7^{o}	1.4^{n}	2.5^{lm}
2	2.4^{m}	2.3^{m}	3.1^{l}	7.0^{g}
3	4.2^{j}	4.2^{j}	3.5^{kl}	7.7^{ef}
4	6.1^{h}	7.4^{fg}	3.8^{jk}	8.1^{de}
5	6.4^{h}	9.9^{a}	5.0^{i}	8.3^{cd}
6	6.3^{h}	10.4^{a}	5.2^{i}	8.7^{bc}
7	5.9^{h}	8.5^{cd}	6.3^{h}	9.2^{b}

[1]Superscript letters indicate that a value followed by the same letter did not differ significantly ($P<0.05$) in Duncan's multiple range test (DMRT) from other values with the same letter

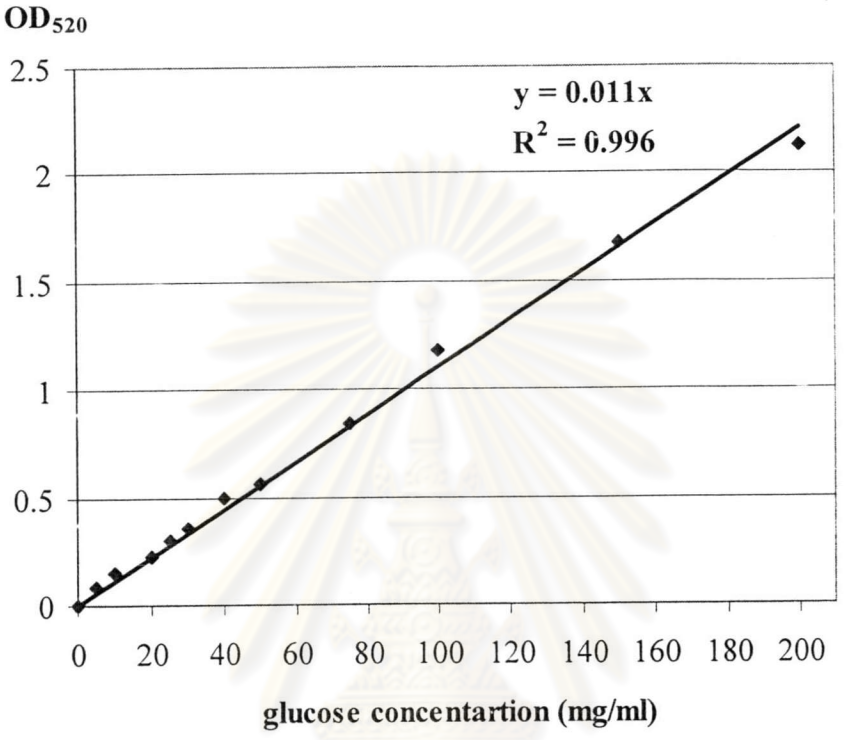
ANOVA

EPSs from SK3	Sum of Squares	df	Mean Square	F
Between Groups	637.631	27	23.616	192.770*
Within Groups	6.860	56	.123	
Total	644.491	83		

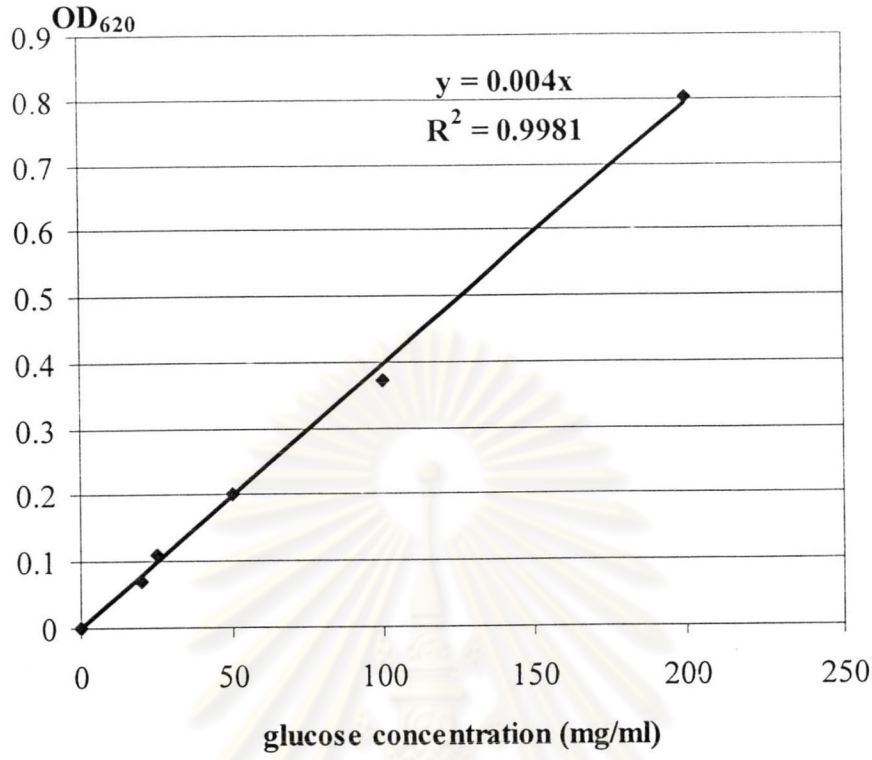
* = Significant at 95%

APPENDIX III

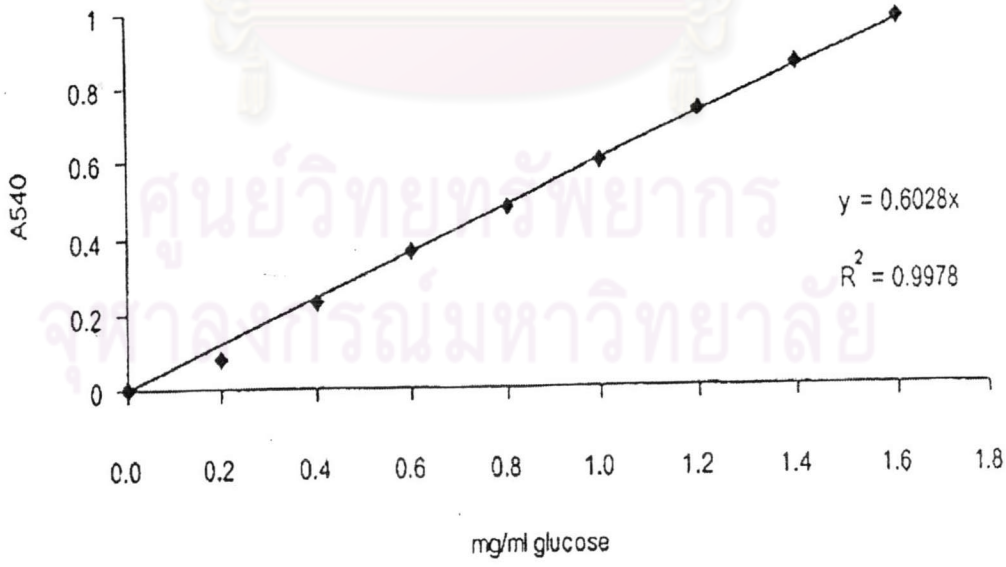
Standard curve for amylase and pullulanase activity assay



Standard curve for anthrone analysis



Standard curve for pullulanase sensivivity test



APPENDIX IV

Using a hemacytometer to determine total cell counts

1. With the cover-slip in place, use a Pasteur pipette or other suitable device and transfer a small amount of cell suspension to both chambers of the hemacytometer by carefully touching the edge of the cover-slip with the pipette tip and allowing each chamber to fill by capillary action. Do not overfill or under the chambers.
2. Starting with 1 chamber of the hemacytometer, count all the cells in the 1 mm center square and four 1 mm corner squares. (see Diagram I)

   Note: Count cells on the top and left touching middle line of the perimeter of each square. Do not count cells touching the middle line at bottom and right sides. (see Diagram II).

3. Repeat the procedure for chamber 2.

   Note: If greater 10% of the cells appear clustered, repeat entire procedure making sure the cells are dispersed by vigorous pipetting in the original cell suspension. If less than 200 or greater than 500 cells (20-50 cells per square) are observed in the 10 squares, repeat the procedure adjusting to an appropriate dilution factor.

4. Cell Counts-Each square of the hemacytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 in equivalent to 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculation:

CELL PER ml = the average count per square x dilution factor x 10^4 (count 10 squares)

Ex: If the average count per square is 45 cells x 104 = 4.5 x 10^5 cell/ml.

TOTAL CELLS = cells per ml x the original volume of fluid which cell sample was removed.

Ex: 4.5 x 10^5 (cell per ml) x 10 ml (original volume) = 4.5 x 10^8 total cells

5. Withdraw a second sample and repeat counting procedure to ensure accuracy.

DIAGRAM I

STANDARD HEMACYTOMETERCHAMBER

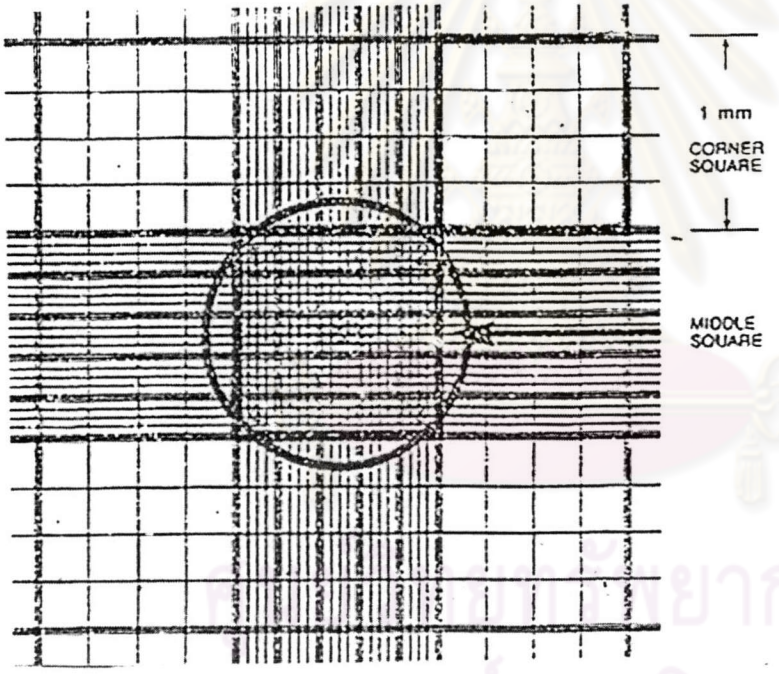
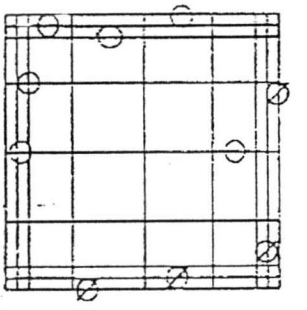


DIAGRAM II

CENTERSQUARE

(ENLARGEMENT)



Count cell on top and left
Touching middle line(O)
Do not count cells touching
middle line at bottom and right.

The circle indicate the approximate area converted at 100x microscope magnification (10X ocular and 10X objective) Include cell on top and left touching middle line (O).

Do not count cells touching middle line bottom and right (Ø). Count 4 corner squares

and middle square in both chambers (one chamber represented here).

BIOGRAPHY

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Dissertation title: "Characterization of exopolysaccharide produced by *Aureobasidium pullulans* isolated in Thailand"

Research collaboration: Department of Biochemistry and Microbiology, Rutgers University, NJ, USA and Biopolymer Research Unit, Agricultural Research Service, USDA, Peoria, IL, USA

Research publications and presentations:

1. S. Prasongsuk, R.F. Sullivan, M. Kuhirun, D.E.Eveleigh, and H. Punnapayak. 2005. Thailand habitats as sources of pullulan producing strains of *Aureobasidium pullulans*. World Journal of Microbiology and Biotechnology (In press).

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