PURIFICATION OF SIDEROPHORES FROM PINEAPPLE (ANANAS COMOSUS)

THEERAWAT SANKUMLOR

Department of Chemistry, Faculty of Science, Chiang Mai University, Thailand
E-mail: thsa44@gmail.com

Abstract- Siderophores are important natural iron chelator. They are used as one of the commercial drugs for human blood-iron excess disease **(**Thalassemia**)** treatment. This chelating compounds have been founded only in some graminaceous plants, fungi and most rhizospherical micro-organisms under condition of iron-deficiency. Nevertheless, primary tests of aqueous extracts from each part, e.g., top, slip or leaf of pineapple **(**Ananas comosus**)** showed positive results of iron chelating ability with chrome azurol s **(**CAS**)** solution test. Isolation and purification of the active components revealed that its siderophores are hydroxamate type mixed with catecholate. This is the first report on siderophore in the bromeliads which is non-gramineceous plant's family.

Keywords- Siderophore; Iron chelator; Pineapple (Ananas comosus); Waste; Extraction; Isolation; Purification; Partial identification; Chrome azurol s (CAS)

I. INTRODUCTION

In nature, most important iron forms for life's bioactivities can be found at least in two forms, e.g., ferrous form (Iron II) and ferric form (Iron III) [1]-[2]. Ferrous form can easily dissolved in water via anaerobic condition of near earth crust surface. Thus it can be easily taken up by any of lifeform [3]. While ferric form is insoluble with water via aerobic condition beneath the soil surface where plant root lied. Thereby ferric form is harder to be uptaken and used than ferrous from [3]-[4].

Unfortunately, most ferrous form need to be changed into ferric form at last when used by any metabolism process parts, i.e., creation of plant's chlorophyll and core of animal's heme [5]-[9] then contained in tissue of living body. Losing in right balance level of iron especially ferric form can caused many dangerous sickness as an example of human blood-iron excess (Thalassemia). Using chelator as a drug was one of a good treatment for controlling streaming iron level by carried it in and out.

Siderophores are one of well natural iron chelating compounds. Generally, they can be divided into at least 3 types, i.e., hydroxamates, catecholates and mixed. In medicinal aspect, siderophores are now used as one of the drug for Thalassemia [10]-[12]. It caused very high expenses. Not only because of very long-term usage like genetically lifetime case. However, also production source limits. Siderophores have founded only by some graminaceous plants, fungi and most bound of rhizospherical micro-organisms [13]-[17]. They released siderophores while under iron-deficiency condition. In Thailand, pineapple (Ananas comosus) is one of the most tolerance plants. It was cultivated in every regions [18]-[19] even in the sliding red soil that containing highly unavailable iron and nutrients. For some site, it even planted without any taking care. That indicated there was very high uptaking ability of nutrients and iron. Moreover, they gave many tons of wastes and by-products, e.g., top, slip and leaf [20] after each yearly harvest. Hence, each parts of the plant were studied in order to find siderophores employing chrome azural s (CAS) solution testing reagent, chromatography, spectroscopy and spectrometry techniques.

II. MATERIALS AND METHODS

A. Preparation

Parts of fresh pineapple plant, e.g., tops, slips and leaves were sampled from pineapple farms in Chiang Rai and Chiang Mai provinces of northern Thailand. Each parts of the plant were cut into small pieces before dried with optimized conditions at constantly temperature 50°C and held over 15 to 24 hours. Next, dried plant parts were grinded then warped and kept from light under nitrogen atmosphere as raw material. This could extend its expire date to over 6 mount or even 1 year at room temperature about 20 to 25 °C.

Solvents and chemicals including all commercial, analytical and GC-HPLC grades were purchased from Merck, Germany. Also silica gels on aluminum clothed plate for thin-layer chromatography (TLC) and silica gels for column chromatography (CC) were purchased from Merck KGaA, Germany. All consumable stuff and packed columns for high performance chromatography were brought from Hewlett-Packard, U.S.A. Calibration standard like C8-C40 Alkanes was purchased from Sigma-Aldrich, U.S.A. which was now subsidiary of Merck.

B. Extraction

Raw material from pineapple plant parts of all varieties were extracted by maceration, infusion, decoction and soxhlet extraction methods.

For maceration, raw material submerged in each solvent, e.g., hexane, dichloromethane, ethyl acetate, methanol ethanol and water then kept within room

temperature for 24 hours and repeated 3 times. Infusion was held by a set of refluxing instrument. Raw material immerged in each solvent while gave temperature between 50 °C and 80 °C for 15 minutes before repeated 3 times. Decoction was infusion alike but increased a higher temperature range until reached water boiling point amidst 90 °C and 110 °C for 10 minutes, also repeated 3 times. Soxhlet extraction was carried out with the combination of siphon technical apparatus and refluxing of indirect solvent that only pass through raw material but not drown it all the time. This made it reached but not over each solvent boiling points. After the first reflux through the material, it was held by 45 minutes then changed only solvent and repeated again 3 times.

Each time of repeating the methods, solvents evaporated out of the extracts. Crude extracts were kept separately before had a reunion with the next round of repetition which used a new set and finally counted as a total yield of each method.

After the extractions, all extracts were tested with chrome azurol s (CAS) solution which modified from assay described by Schwyn and Neilands (1987) [21]. The most reasonable variety one was selected and useful method was considered from its own conditions, yield and tested results.

The selected extract then were re-extracted by liquid-liquid extraction (LLE) and gave clearer separation by setting about 8 hours and stirring every 2 hours before repeated LLE until reach 3 times.

C. Isolation and Purification

Selected extract from LLE was loaded to series of silica gels packed column chromatography (CC) therewith elution by solvents systems from determination varied from binary to tertiary systems and collaborated by ultraviolet-visible (UV-Vis) spectroscopy employing UV-Vis spectrophotometer - Lambda 25; PerkinElmer, U.S.A. and fixed wavelength 254 and 365 nm lamps with darkroom viewing cabinet to isolated compounds. Moreover, for clarified partition pattern, phosphomolybdic acid (PMA) solution, potassium permanganate ($KMnO_4$) solution and iodine (I_2) were used as staining technique reagents to increased visibility of compounds. Collection out of column separated to fractions that then were checked by thin-layer chromatography (TLC) with CAS testing concurrently to targeted siderophores. Therefrom CAS positive fraction were isolated, purified compounds ensured by gas chromatography (GC) employing Gas chromatograph (GC) - Agilent 6890; Hewlett-Packard, U.S.A. with optimized conditions.

D. Partial Identification

After ALL simultaneous testing, siderophores were identified by CAS assay and infrared (IR) spectroscopy information of the purified compounds were collected by subjected to IR spectrophotometer - Tensor 27; Bruker, Germany. Partial structure was elucidated to divided pineapple's siderophore types.

III. RESULTS AND DISCUSSION

E. Extraction

From the extraction results as shown in table I. Decoction used too high temperature so low boiling point solvents could not handle, one of them might fume and leaved very dangerous inhalation to environment. For soxhlet extraction, even it had higher yield on some solvents especially low boiling point solvents but for high boiling point and important polar solvents, it gave the same or even lower yield. Moreover, it still reached the high boiling point which enough to changed color of extract that might meaning it started to crumble and leaded to losing of some possible compounds.

By now, maceration and infusion leaded good choices. They had close to high yield size while used very low temperature. However, it seems that maceration used so much time and leaved the material submerged. In which for some solvents especially polar solvent like water, it leaded to decaying and wasted the materials those are chance of foods and industrial problems afterward. Therefore, infusion that used not too high temperature with very short period of time was an efficiency method.

TABLE I. RESULTS OF EACH EXTRACTION METHODS

Solvent/ Method		Maceration	Infusion	Decoction	Soxhlet
Hexane	*a*	35.34	34.65	-	40.65
	b	X	X	-	X
Dichloromethane	*a*	18.11	18.09	-	19.96
	b	/	/	-	/
Ethyl acetate	*a*	14.01	14.62	15.53	14.76
	b	/	/	/	/
Ethanol	*a*	23.84	24.36	26.02	19.88
	b	/	/	/	/
Methanol	*a*	15.72	15.55	-	16.94
	b	/	/	-	/
Water	*a*	18.09	17.47	17.93	15.86
	b	/	/	-	-
Temperature (°C)		Room temp.	50-80	90-110	Not over each solvent's boiling point temp.
Time (c) per round		24 hr.	15 min.	10 min	45 min

a = Total yield % (w/w).

b = CAS Test: / (Positive), X (Negative) and - (Not tested).

c = At least 3 times for each methods.

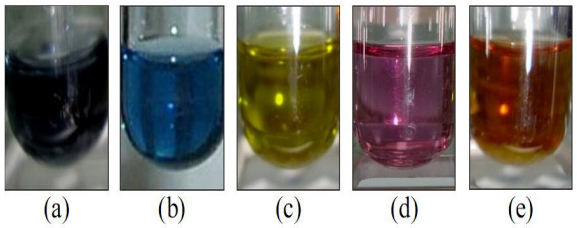


Fig. 1. CAS Test: CAS (a), CAS + solvent (b), sample (c), negative result (d) and positive result (e).

All extracts from infusion were tested iron chelation ability by CAS result in clear color as in figure 1, blue color of CAS changed to orange for positive result and changed to purple for negative one. The results in table I shown that except hexane extract which used very non-polar solvent and gave negative result, even all other solvents gave positive result, dichloromethane and ethyl acetate still could extract only low to middle components so not all siderophores which is water soluble like could come out. While water extract way too high polar and hard to separated, its crude could not be kept for too long in only simple condition. Even ethanol and methanol both seemed good for yield, separating and keeping, ethanol had wider range of extraction than the other so ethanol took the better part to go on.

The selected ethanol extract re-extracted by LLE and gave clearer separation results in table II. It shown that only dichloromethane and ethyl acetate extracts gave positive results. However, dichloromethane extract had higher yield and easier to re-dissolved. For water extract could not be test because its color changed to really viscous dark brown so incomparable.

TABLE II. YIELD PERCENT AFTER LIQUID-LIQUID EXTRACTION

Solvent	Hexane	Dichloro methane	Ethyl acetate	Water
% (w/w) Yield	26.09	17.95	12.4	43.52
CAS Test[a]	X	/	/	-

[a] CAS Test: / (Positive), X (Negative) and - (Not tested).

F. Isolation and Purification

The dichloromethane extract after LLE loaded to packed CC with employed TLC checking for eluents solvent system selection and separating pattern for partition concurrently. Main system was binary of dichloromethane : methanol = 9 : 1 and variation gradient started with hexane and dichloromethane from almost 100% to decreasing at the same period as increasing of % ethyl acetate until 100% then turned the circle process again with ethanol and methanol to not over 10% and held before all in the end drained, respectively.

Isolation path lines shown as figure 2-4. Each level box means one column in the sequence. Code name of each level box; C = column and F = fraction. Each first row in one level was loaded sample. Number next to C = number of column in different level (1 = first order) while number next to F = number of collected fraction (1 = first order) and number between C and F = number of column in the same level (subsequence or branch from the same previous column above). % recovery was summation of weight of all collected fractions compared with weight of loaded sample while % yield was weight of active or positive result with CAS test fraction compared with weight of loaded sample.

Every column levels and fractions were tested by CAS. Path line sequence with 5 columns of first isolated CAS positive compound (AC-S1) shown as figure 2, path line sequence with 6 columns of second isolated CAS positive compound (AC-S2) shown in figure 3 and path line sequence with 5 columns of third isolated CAS positive compound (AC-S3) shown in figure 4.

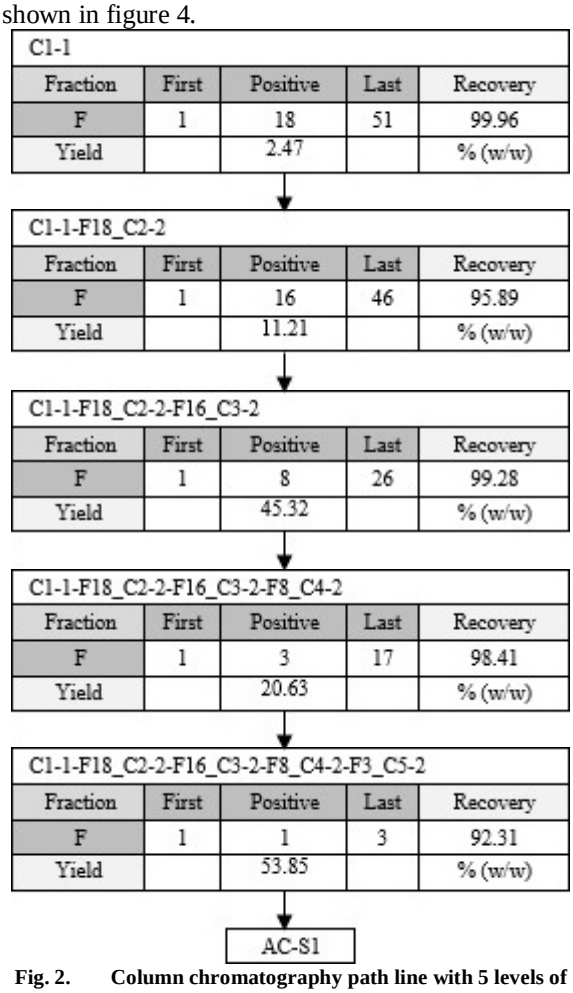
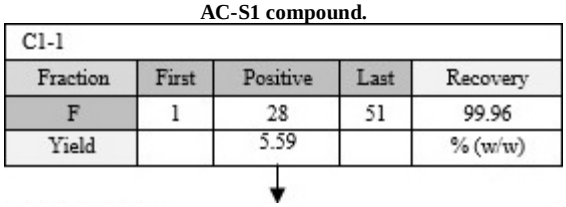


Fig. 2. Column chromatography path line with 5 levels of AC-S1 compound.



C1-1-F28_C2-3				
Fraction	First	Positive	Last	Recovery
F	1	34	48	99.95
Yield		12.30		% (w/w)

↓

C1-1-F28_C2-3-F34_C3-5				
Fraction	First	Positive	Last	Recovery
F	1	19	23	98.26
Yield		44.06		% (w/w)

↓

C1-1-F28_C2-3-F34_C3-5-F19_C4-9				
Fraction	First	Positive	Last	Recovery
F	1	16	17	99.34
Yield		33.55		% (w/w)

↓

C1-1-F28_C2-3-F34_C3-5-F19_C4-9-F16_C5-12				
Fraction	First	Positive	Last	Recovery
F	1	5	7	98.04
Yield		25.49		% (w/w)

↓

C1-1-F28_C2-3-F34_C3-5-F19_C4-9-F16_C5-12-F5_C6-5				
Fraction	First	Positive	Last	Recovery
F	1	5	5	97.14
Yield		38.10		% (w/w)

↓

AC-S2

Fig. 3. Column chromatography path line with 6 levels of AC-S2 compound.

C1-1				
Fraction	First	Positive	Last	Recovery
F	1	36	51	99.96
Yield		5.11		% (w/w)

↓

C1-1-F36_C2-5				
Fraction	First	Positive	Last	Recovery
F	1	35	35	99.96
Yield		8.41		% (w/w)

↓

C1-1-F36_C2-5-F35_C3-11				
Fraction	First	Positive	Last	Recovery
F	1	26	29	96.76
Yield		51.85		% (w/w)

↓

C1-1-F36_C2-5-F35_C3-11-F26_C4-19				
Fraction	First	Positive	Last	Recovery
F	1	9	12	98.21
Yield		72.32		% (w/w)

↓

Fig. 4. Column chromatography path line with 5 levels of AC-S3 compound.

Three active compounds were then confirmed purity by GC with optimized conditions of column HP inno-wax with 60 m × 0.32 mm x 0.25 µm of film thickness. Inlet temperature was 230 °C while transfer line or detector temperature was 240 °C. Injection mode was pretended as splitless and injected volume of 1 µL with carrier gas at constant flow of purified helium at 1.2 ml/minute. Oven programmed 40 °C initially then increased to 240 °C at 5 °C/minute and held over 30 minutes for a total run time of over one hour.

By co-calibration with comparable standard n-alkanes C8-C40, Index which was presented by Kovats (1958) [22] and later IUPAC (1997) [23] can carried out as Kovats retention indices for new unknown siderophore compounds peaks of AC-S1, AC-S2 and AC-S3 in which were 2037, 2964 and 3299, respectively.

G. Partial Identification

Purified compounds were run by solution and thin-film techniques on Fourier transformed IR (FT-IR). Data were collected and peak bands were assigned as figure 5.

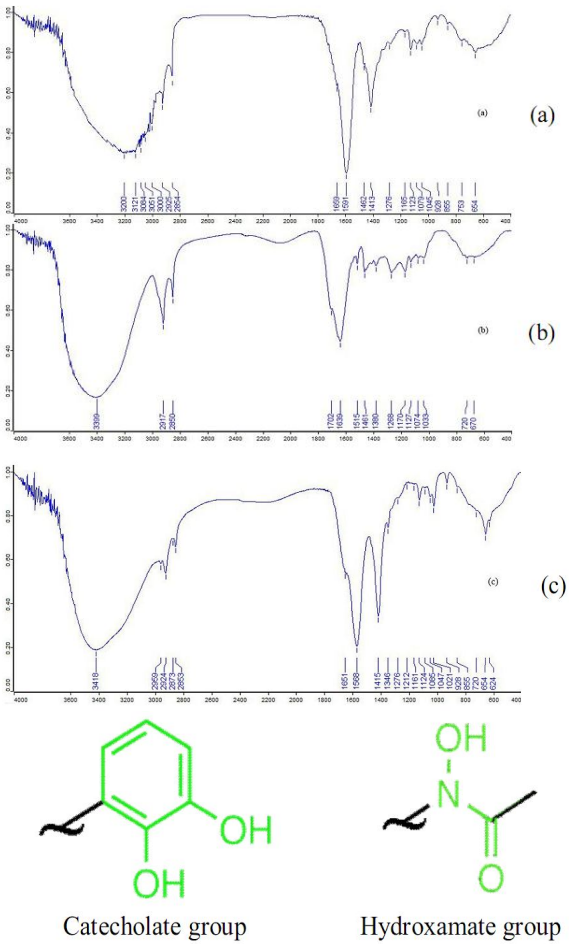


Fig. 5. FT-IR Spectra: spectrum of AC-S1 (a), AC-S2 (b) and AC-S3 (c).

Broad band peaks in spectrum of AC-S1 around 3200 from over 3600 to 2850 cm^{-1} had contributions from OH with traced side of aromatic–OH (Ar–OH)

stretching as phenolic group. Also absorption at 1714 and 1204 cm^{-1} are contributed from C=O and C–O stretching in –O=C-O and Ar–OH. Peak at 1659 to 1591 cm^{-1} could be assigned to C=O of carbonyl group. Breathing CH2 of aromatic ring was down at beneath 1462 cm^{-1}. Series around 753 cm^{-1} might be aromatic ring CH plane binding.

AC-S2 spectrum shown functional OH and NH groups presented in broad regions around 3600-3200 cm^{-1} like AC-S3. The strong 1750-1550 cm^{-1} was assigned as >C=O and >CN from NH–C=O vibrational coupled. The absorption at 1450-1330 cm^{-1} and around 1050 cm^{-1} might be CH3 bend while the peaks between 1320 cm^{-1} and 1000 cm^{-1} were attributed to C–N and N–O stretching.

Spectra of the compounds seem shown similarity. However, trace of different structures also revealed. For AC-S1, the stronger carbonyl peak over band around 3000 cm^{-1} of Ar–OH shown highly clean of cartecholate siderohore type. While the other two had a different but seem related to each other. Two of them had a little shifter both hydroxyl band and carbonyl peak to revealing the presence of structural nitrogen of hydroxamate group drifted from AC-S1. However, AC-S2 had a stronger separately band of above 3200 cm^{-1} over carbonyl than AC-S3. This might because of some trace of another different functional group included in the structure, according to undefined aromatic characteristic side, absorption over 2950 cm^{-1} and 1400 cm^{-1} might be C–H and C=O bending due to the influence of O–H stretching or aromatic itself, AC-S3 could be assigned as mixed type siderophore.

CONCLUSION

Positive CAS testing results revealed that pineapple (Ananas comosus) had iron chelation ability of siderophores.

Double extraction by infusion then LLE can save both time and energy usage. That also prevented raw material from direct contraction to any poisons on isolation and purification process and the extracted materials still can be used with less environmental impact for another feed or industrial proposes.

Moreover, the isolation and purification process can be repeated and tracked pathway lines.

Consider identified results, it had at least three types of siderophores. Dividing by its presence of major functional groups in AC-S1, AC-S2 and AC-S3 structures, the siderophores could be assigned as catecholate, hydroxamate and mixed types, respectively.

By the way of collection, materials, extracts and siderophore compounds can be kept long enough to being applicate. These can be regarded as adding value to this economic crop, reducing its wastes and maximizing its benefits.

ACKNOWLEDGMENTS

Gratefully appreciations to my family, advisor, teachers and friends for every supports. Proudly thank to Department of Chemistry, Faculty of Science, Chiang Mai University, Thailand for laboratory and instruments. Likewise thanks to Department of Biochemistry and Industrial Chemistry, Faculty of Science, Chiang Mai University,

Thailand for instruments. Besides thanks to Faculty of Agriculture, Chiang Mai University and Pineapple field, Mae Chan, Chiang Rai, Thailand for materials and information with moreover thank to Pineapple field, Mae Suai, Chiang Rai, Thailand for materials.

REFERENCES

[1] R.M. Cornell, U. Schwertmann, “The Iron Oxides: Structure, Reactions, Occurrences and Uses,” New York Wiley-VCH, 2003, 2, pp. 1-7.

[2] T.D. Jickells, et al., “Global Iron Connections Between Desert Dust, Ocean Biogeochemistry, and Climate,” Science, 2005, 308, pp. 67-71.

[3] L. Pérez-Guzmán, K.R. Bogner, B.H. Lower, “Earth’s Ferrous Wheel. Nature Education Knowledge,” Nature Education Knowledge, 2010, 3, 10, 32.

[4] F. von Blanckenburg, N. von Wiren, M. Guelke, D.J. Weiss, T.D. Bullen, “Fractionation of Metal Stable Isotopes by Higher Plants,” Elements, 2009, 5, 6, pp. 375-80.

[5] H. Fones, G.M. Preston, “The impact of transition metals on bacterial plant disease,” FEMS Microbiol, 2013, 37, pp. 495-519.

[6] S.J. Lippard, J.M. Berg, “Principles of Bioinorganic Chemistry,” Mill Valley: University Science Books, California, 1994, pp. 411.

[7] R.S. Ajioka, J.D. Phillips, J.P. Kushner, “Biosynthesis of heme in mammals,” Biochimica et Biophysica Acta, 2006, 1763, pp. 723-36.

[8] A.C. Dlouhy, C.E. Outten, “The Iron Metallome in Eukaryotic Organisms,” Metal Ions in Life Sciences, 2013, 12, pp. 241-78.

[9] G.M. Yee, W.B. Tolman, “Transition Metal Complexes and the Activation of Dioxygen,” Metal Ions in Life Sciences, 2015, 15, pp. 131-204.

[10] A. Cao, V. Gabutti, G. Masera, B. Modell, G. Sarchia, C. Vullo, “Management protocol for the treatment of thalassaemia patients,” Newyork Cooly’s Anaemia Foundation, 1992.

[11] A. Kolnagou, G.J. Kontoghiorghes, “Advances in the prevention and treatment are changing thalassemia from a fatal to a chronic disease. experience from a Cyprus model and its use as a paradigm for future applications,” Hemoglobin, 2009, 33, 5, pp. 287-95.

[12] U. Möllmann, L. Heinisch, A. Bauernfeind, T. Köhler, D. Ankel-Fuchs, “Siderophores as drug delivery agents: application of the ‘Trojan Horse’ strategy,” Biometals, 2009, 22, 4, pp. 615-24.

[13] E.A. Buxton, B.G. Hopkins, V.D. Jolley, R.C. Christensen, “Phytosiderophore exudation from the roots of iron stressed Kentucky bluegrass,” West. Nutri. Manage. Conf., 2009, 8, pp. 68-71.

[14] I. Tolay, B. Erenoglu, V. Römheld H.J. Braun, I. Cakmak, “Phytosiderophore release in Aegilops tauschii and Triticum species under zinc and iron deficiencies,” Journal of Exp. Botany, 2001, 52, 358, pp. 1093-9.

[15] A. Koulman, T.V. Lee, K. Fraser, L. Johnson, V. Arcus, “Identification of extracellular siderophores and a related peptide from the endophytic fungus Epichloë festucae in culture and endophyte-infected Lolium perenne,” Phytochemistry, 2012, 75, pp. 128-39.

[16] H.P. Fiedler, P. Krastel, J. Muller, K. Gebhardt, A. Zeeck, "Enterobactin: the characteristic catecholate siderophore of Enterobacteriaceae is produced by Streptomyces species," FEMS. Microbio. Letters, 2001, 196, pp. 147-51.
[17] S. Dhungana, R. Michalczyk, H. Boukhalfa, J.G. Lack, A.T. Koppisch, "Purification and characterization of rhodobactin: a mixed ligand siderophore from Rhodococcus rhodochrous strain OFS," Biometals, 2007, 20, pp. 853-67.
[18] "Pineapple and Products," Department of Foreign Trade, Ministry of Commerce, Thailand, 2012.
[19] U.M. von der Linden, "Pineapple juice and concentrate – a versatile and complex tropical submarket," Inter. Fruit Juice, 2005, 45.
[20] S. Ketnawa, P. Chaiwut, S. Rawdkuen, "Pineapple wastes: a potential source for bromelain extraction," Food and Bioproducts Processing, 2012, 90, pp. 385-91.
[21] B. Schwyn, J.B. Neilands, "Universal chemical assay for the detection and determination of siderophores," Anal Biochem, 1987, 160, pp. 47-56.
[22] E. Kovats, "Gas-chromatographische Charakterisierung organischer Verbindungen. Teil 1: Retentionsindices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone," Helv. Chim. Acta, 1958, 41, 7, pp. 1915-32.
[23] IUPAC., "Retention index, I in column chromatography," Compendium of Chemical Terminology, the Gold Book, 1997, 2.

★★★