International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2018): 7.426

Effect of Solvents on the Production of N-Acetyl Glucosamine as a Result of Enzymatic Hydrolysis of Chitin from Shrimp Shell Waste

Nuniek Herdyastuti[1], Sari Edi Cahyaningrum[2]

Department of Chemistry, Universitas Negeri Surabaya, Surabaya – East Java Indonesia

Abstract: ***N-Acetyl glucosamine (GlcNAc) or 2-acetamido-2-deoxy-D-glucose is a simple amino sugar that is a monosaccharide that has an amino group in its structure. GlcNAc is very potential to be used in industry because it has many significant applications and has high efficiency. GlcNAc can be obtained by chemical hydrolysis or enzymatic degradation of chitin compounds using the enzyme chitinase. o get GlcNAc it is necessary to carry out enzymatic isolation of chitin, purification using several solvents and characterization. This study aims to look at the effect of solvents on the production of N-Acetyl glucosamine which has been hydrolyzed from chitin enzymatically with the enzyme chitinase. The N-acetyl glucosamine produced was determined by HPLC, XRD and IR-Spectrophotometer. Purification results with ethanol, methanol, acetone and acetonitrile solvents give chromatogram results at a retention time of about 3.1. FT-IR results indicate the presence of functional groups N - H, C - H, O - H and C = O which are also found in standard N-Acetyl glucosamine. The crystallinity test results showed a major peak of 2Θ ie 10; 15, 20 and 30. Purification using ethanol solvent showed the highest purity results compared to other solvents and also showed physical and chemical characteristics that were close to standard N-acetyl glucosamine.***

Keywords: Enzymatic hydrolysis, chitin, N-Acetyl glucosamine

1. Introduction

Chitin is a polymer that is very abundant in nature and ranks second after cellulose. Chitin is a linear form of polysaccharides formed from β-1.4 residues of N-acetyl-glucosamine residues. Disaccharides from monomers of the compound N-acetyl-D-glucosamine are often referred to as chitobiose and the bonds to these molecules form linear fibra [1]. N-acetyl glucosamine or 2-acetamido-2-deoxy-D-glucose (GlcNAc) is said to be a simple amino sugar that is a monosaccharide which has an amino group in its structure. The amino monosaccharide has the molecular formula $C_8H_{15}NO_6$, and its molecular weight is 221.2. Chen and Liu (2010) stated that in general N-acetyl-glucosamine is white and slightly sweet powder, has a melting point at 221°C, has a 25% solubility in water, is colorless and clear [2]. The same was said by Windhyastuti (2010) that N-acetylglucosamine is a white powder which has a slightly sweet, odorless taste, with a melting point of 221°C and is soluble in water with 25% solubility, slightly soluble in methanol which is heated and insoluble in diethylether [3]. N-Acetylglucosamine (GlcNAc) has the molecular formula $C_6H_{15}NO_6$that contains 6.9% pure nitrogen mixture with chemical structurewhich is the same as cellulose by an acetyl amino unit(CH_3COONH_2). N-acetylglucosamine canenzymatically produced and generally N-asetylglucosaminecan produced through acid hydrolysis (HCl)from Chitin. GlcNAc is generally in the form of white powder withsweet taste so it is used as a substitute for sugar andreceived great attention in osteoarthritis [4].N-acetylglucosamine has a functional group that is almost the same as chitin because chitin has an acetamide group ($NHCOCH_3$) so that chitin is a polymer with N-acetylglucosamine unit, the difference is that chitin has a CH_2OH group in atom C number 5 while in N-acetylglucosamine there is only OH group.

N-acetyl glucosamine can be produced enzymatically by the addition of the enzyme chitinase to chitin and non-enzymatic by adding acidic compounds such as sulfuric acid or hydrochloric acid to chitin degradation. Every year about 100 billion tons of chitin are produced in nature so chitin can be used as a suitable biomass resource for producing N-Acetyl glucosamine through a process based on hydrolysis of chitin [2].

Enzymatic hydrolysis of chitin has been carried out using chitinase enzymes derived from chitinolytic bacteria such as Pseudomonas sp TNH 54. N-Acetyl glucosamine has very good potential in both the health and industry sectors. Some of the potential of N-Acetyl glucosamine include being able to function as a nutrient, an intermediate metabolite and is required for cell function. N-acetyl D-glucosamine and D-glucosamine are food supplement candidates, and act as a treatment in patients with osteoarthritis [5]. Medical research involving N-acetylglucosamine shows potential for various treatments for autoimmune diseases using glucose derivatives. The presence of N-acetylglucosamine in the thymus gland also seems to prevent the formation and growth of abnormal thymus cells (T-cells), which contribute to autoimmune disorders [6]. Recent research has also been carried out that N-acetyl-glucosamine and its derivatives have been used in food supplements and for therapeutic development. Toxicity test results reveal that N-acetyl-glucosamine is non-toxic, safe for the body and further shows that 54% of the glucosamine given will be excreted into the urine in one day.

Solvents fulfill several functions in chemical reactions, where the solvent dissolves the reactants and reagents so that they mix, so this will facilitate the integration of the reactants and reagents that should occur in order to convert the reactants into products. The solvent also acts as a temperature control, one of which is to increase the

energy of the particle collision so that the particles can react faster, or to absorb the heat generated during an exothermic reaction. In general, a good solvent has the following criteria: (1) the solvent must not be reactive (inert) to reaction conditions, (2) the solvent must be able to dissolve the reactants and reagents, (3) the solvent must have the right boiling point and 4) The solvent must be easily removed at the end of the reaction. The second criterion is to use the principle of like dissolves like, where nonpolar reactants will dissolve in nonpolar solvents while polar reactants will dissolve in polar solvents.

2. Experiment

2.1 Material and Methods

Commercially acquired chitin (Rhongseng, China), which is then made into an amorphous form according to Illankovan. Chitinase enzyme is produced from the bacterium *Pseudomonas sp* TNH54 and separated by centrifugation (Eppendorf 5810 R). N-acetyl glucosamine is produced from the hydrolysis of amorphous chitin with the enzyme chitinase. Purification of N-acetyl glucosamine with solvents was identified by HPLC (Hewlet Packard Series 1050), Spectrophotometer-FTIR (Perkin Elmer) and XRD (Bruker type D8 Advance) and has been compared with N-Acetyl glucosamine standard.

2.2 Chitinase production

Chitinase enzymes produced from a single colony of the bacterium *Pseudomonas sp* TNH54 were grown on 100 mL liquid screening media containing 0.4% chitin at room temperature with shaking 120 rpm for 20 hours. The liquid culture was then centrifuged at 8000xg for 15 minutes at 4ºC. The supernatant obtained was a crude extract of chitinase.

2.3 Synthesis of amorphous chitin

10 g of chitin were dissolved in a mixture of 40% NaOH solution and 0.2% SDS (which had been cooled at 4ºC) which the solution is swelled for 1 hour at 4°C.Chitin slurry matrix was stored for 1 night at a temperature of -20ºC, then neutralized with HCl 6 N. Then filtered, and washed in the order of solvent ethanol, water, ethanol, and acetone. The results are dried with a freeze dryer, amorphous chitin is obtained.

2.4 Production of N-Acetylglucosamine

2 mL of 1.2% (w/v) chitin solution was dissolved in 200 mM potassium phosphate buffer stirred with a magnetic stirrer and 0.5 mL of chitinase enzyme solution was added. Furthermore incubated for 8 hours at room temperature with shaking speed of 120 rpm. After 8 hours the mixture is placed into boiling water for 5 minutes and cooled at room temperature. The suspension was centrifuged for 10 minutes at 4000 rpm and the supernatant obtained was N-acetyl glucosamine.

2.5 Effect of Solvents on Purification of N-Acetyl Glucosamine

N-acetyl glucosamine obtained from hydrolysis of chitin is added with a solvent (ethanol, methanol, acetone and acetonitrile). The mixture was separated and the precipitate obtained was then dried in an oven at 50 ºC and tested using HPLC (HP 1050) in the Waters column; using a 210 nm UV detector; speed of 1 mL / min; injection of 0.1 mL

2.6 The physical and chemical characteristics of N-Acetyl glucosamine are determined based on color, odor, texture, melting point, moisture content and solubility of water and methanol

3. Result and Discussions

3.1 Production of N-Acetylglucosamine

Chitin can be degraded by enzymatic and non-enzymatic by using strong acids such as HCl, H_2SO_4 atau HNO_3. Hidrolisis kitin menjadi monomernya dapat terjadi dengan cara pemutusan ikatan glikosidik pada ikatan N-asetil. Chitin from shrimp shell which has been modified into amorphous type by adding sodium dodecyl sulphate hydrolyzed enzymatically using the enzyme chitinase from *Pseudomonas sp* TNH 54. The substrate concentration used was 1.2% and incubated for 8 hours to obtain the maximum N-Acetyl glucosamine. Enzymatic degradation of chitin has been widely carried out because it is a simple, fast and reproducible method to produce chitin or chitin oligosaccharide derivatives [7]. Production of N-acetyl glucosamine through acid hydrolysis requires concentrated acid, high temperature, high cost, low yield (below 65%) and acid waste [8], so many focus more on enzymatic degradation of chitin [9,10]. The amount of N-acetyl glucosamine produced is influenced by substrate concentration, enzymes and degradation time used.The N-acetyl glucosamine obtained in the enzymatic degradation process is still yellowish white when compared to commercially obtained N-acetyl glucosamine. This is because in the production process there are still media containing yeast or peptone which are yellow so that the color is bound to N-Acetyl glucosamine. One of them can be done by using activated carbon (Fig 1). Addition of activated charcoal to the solution of hydrolysis products can be done so that changing the color of the original yellow to clear after the process of adding activated charcoal [3].

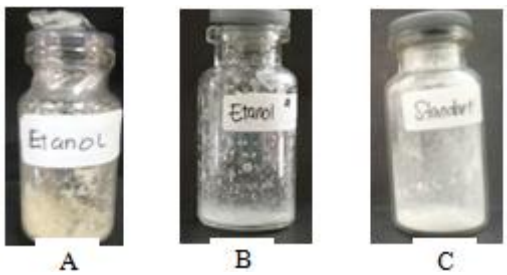

Figure 1: N-acetyl glucosamine from enzymatic degradation of purified enzymes (A), after adding activated carbon (B) and commercially (C)

Other studies explain that the yellowish color in the enzyme solution is caused by the components in the fermentation media such as peptone, yeast extract, malt extract, meat extract, and the sterilization process at 121°C which can play a role in increasing the intensity of the media color. This results in a solution from the yellowish fermentation process and it can be removed using activated charcoal [11, 12]

The results obtained still show a number of blends, so it needs to be purified. The process of purifying N-acetylglucosamine can be carried out by the precipitation method using ethanol. Water, N-acetylglucosamine and ethanol are polar compounds. The addition of ethanol into the N-acetylglucosamine solution resulted in ionic interactions between N-acetylglucosamine with water is reduced because water reacts with ethanol. This causes the deposition of N-acetylglucosamine [1].Some ways that have been done to purify N-acetyl glucosamine are using ion exchange membrane electrophoresis [13]. Zhan (2007) succeeded in finding a new method for purification and recrystallization and succeeded in getting N-Acetyl glucosamine with a purity of 99.95% [14]. Other purification techniques developed are the addition of ozone [15].

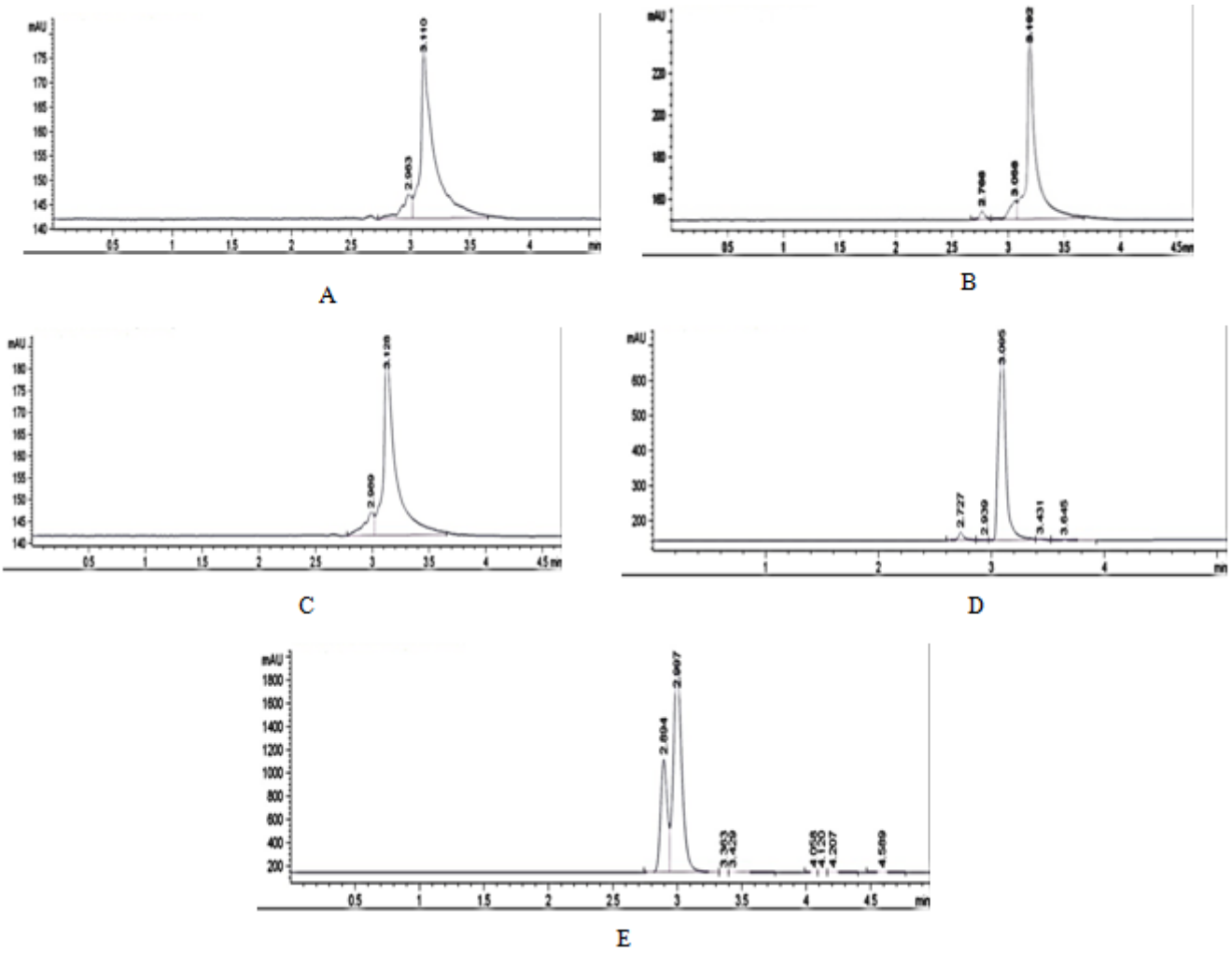

Figure 2: The results of the purification of N-acetyl glucosamine in the four stage with various solvents using ethanol (A), acetonitrile (B),acetone (C), methanol (D) and commercialy NAG (E)

In this research, the solvent was used in stages, namely by adding ethanol which was varied with acetonitrile. The results of the purification of N-Acetyl glucosamine which was analyzed using HPLC showed a peak chromatogram as well as the standard N-Acetyl Glucosamine (Fig 2). When observed in the four types of solvents showed almost the same pattern, where the peak which is suspected to be N-Acetyl glucosamine has been well separated although there are still 1 other peak that has not been separated and is thought to be an N-Acetyl glucosamine oligomer.Separation of two or more substances is based on differences in the distribution coefficient. If one solute has a K value greater than 1 and the other is smaller than 1, then a single extraction can be separated. However, if the two substances have the same but not identical distribution coefficient, the separation will only separate partially, so it is necessary to add another solvent using the Lyman Craig method [17].

N-acetyl glucosamine that has been purified with several solvents has different purity (Table 1). Based on Table 1 shows that N-Acetyl glucosamine produces high purity, this supports the chromatogram of HPLC which shows a peak. Purification using ethanol shows the highest yield

compared to other purifications, besides that purification using ethanol can produce more yields. So that purification using ethanol can be used as an alternative to the purification of N-Acetyl glucosamine.

Table 1: Purity of N-acetylglucosamine in several solvents

N-acetyl glucosamine	Retention time (minute)	Area broad (mAU.s)	Area percentage (%)	Purities (%)
Standart	3.2	369.8	80.8	99
Ethanol purification	3.1	361.3	90.0	97.7
Methanol purification	3.1	357.3	93.9	96.6
Acetone purification	3.1	319.4	90.4	86.4
Acetonitrile purification	3.2	339.9	83.4	91.9

Table 2: N-Acetyl glucosamine characteristics of chemical and phisical properties

No.	N-acetyl glucosamine	Characteristics of phisical properties					Characteristics of chemical properties		
		Colour	smelling	Taste	Texture	Melting point (°C)	Moisture content (%)	Solubility	
								Aquades (%)	Methanol (%)
1.	Standard	White (++)	-	-	powder	218	9.4	95.5	58
2.	Ethanol purification	white (+)	-	-	Fine grain	243	10.9	88.4	30
3.	Methanol purification	Cream	-	-	Fine grain	300	11.0	78.8	35
4.	Acetone purification	Cream	-	-	Fine grain	226	12.1	65.3	40
5.	Acetonitrile purification	Cream	-	-	Fine grain	220	12.4	64.8	42

Based on Table 2 shows that there are some differences in standard N-Acetyl glucosamine with those obtained in enzymatic hydrolysis especially at the melting point. Based on research conducted by Chen et. al., (2010) and Widhyastuti (2010) state that the melting point of N-Acetylglucosamine is in the range 190 - 221 ° C, with 25% solubility in water. Solubility is the ability of a substance to be able to mix perfectly with a certain solvent. Solubility of a compound in a solvent or a number of solvents, in addition to being influenced by polarity is also influenced by the ability to form hydrogen bonds with solvent molecules. N-Acetyl glucosamine is a polar compound that dissolves easily in water, but is slightly soluble in methanol [2,13].

The solubility of N-acetylglucosamine is one of the parameters that can be used as a standard for assessing the quality or quality of N-acetylglucosamine. The higher the solubility of N-acetylglucosamine the better the quality or quality of N-acetylglucosamine. The results of N-acetylglucosamine characterization by FTIR showed spectra of O-H functional groups around 3500 cm^{-1}, C = O around 1670 cm^{-1}, and also C-H at 1378 cm^{-1}. N-acetyl glucosamine from degradation shows a wide peak in the area of 3500 cm^{-1}, this is possible because of overlapping N-H groups and also O-H (Table 3).

Table 3: Analysis of the determination of the functional group N-acetyl glucosamine

N- Acetyl glucosamine	Wave number(cm^{-1})	Functional group
Standart	3467.3	O – H
	2910.5	C – H
	1627.6	C = O
	1329.1	C – N
	1088.3	C – O
Ethanol, methanol and acetone purification	3467.3	O – H
	2910.9	C – H
	1637.4	C = O
	1125.4	C – N
	1062.9	C – O
Acetonitrile purification	3160	O – H
	-	C – H
	1623	C = O
	1089.5	C – N
	1089.5	C – O

N-Acetyl glucosamine with the molecular formula $C_8H_{15}NO_6$ is a white crystalline powder that is soluble in water and has a melting point of 195 - 205ºC. The results of XRD analysis prove that N-Acetyl glucosamine which has been enzymatically hydrolyzed is a crystalline powder that can be identified at the main peak 2Θ, namely 10; 15, 20 and 30 (Fig 3).In N-Acetyl glucosamine hydrolysis results showed a major peak of 2Θ at the same peak, but the purification of ethanol was dominated by a very high peak at 25. Whereas N-Acetyl glucosamine obtained by purification of acetone and acetonitrile is not yet a perfect crystal but is a perfect crystal amorphous shape, but the main peaks still appear at 10, 20 and 30.

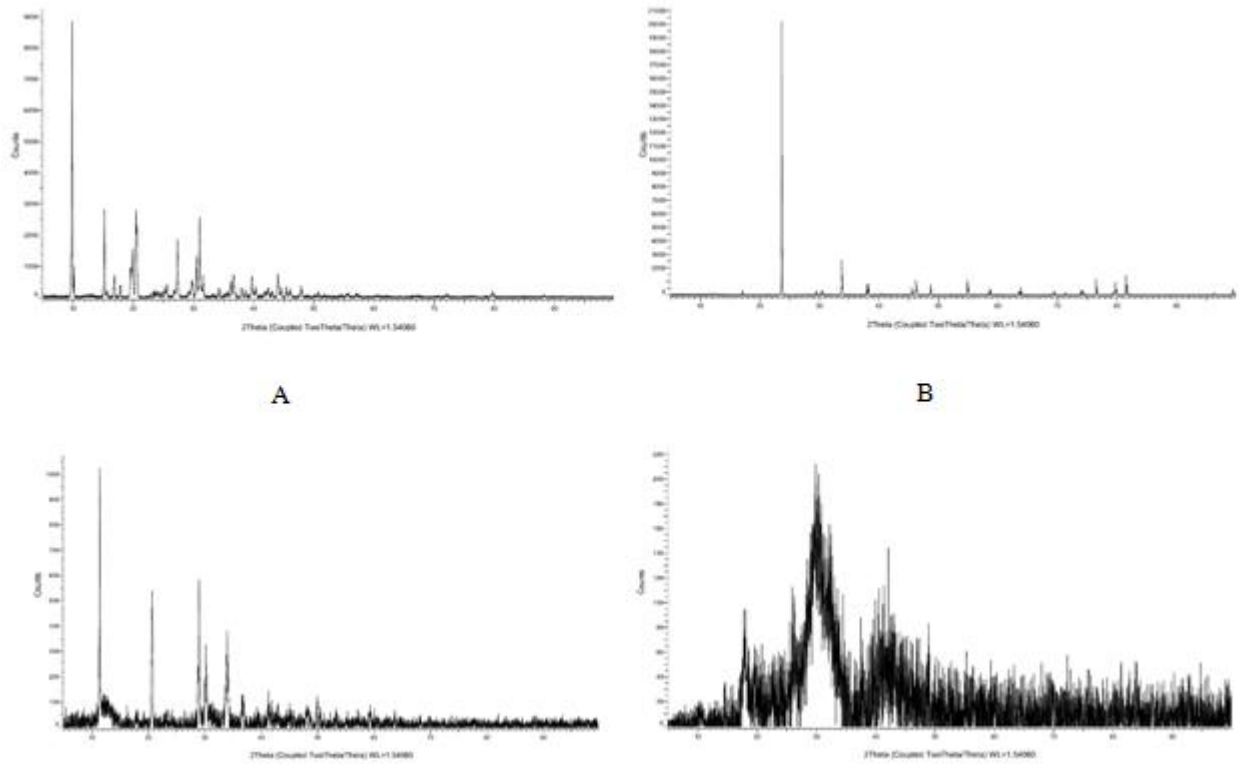

Figure 3: XRD chromatogram results of N-Acetyl Glucosamine standart (A), ethanol purification (B) acetone purification (C) and acetonitrile purification(D)

4. Conclusion

N-acetyl glucosamine obtained from hydrolysis enzymatically can be purified using ethanol, methanol, acetone and acetonitrile solvents. Purification results using ethanol showed the highest purity and characteristics close to N-acetyl glucosamine standard.

Acknowledgement

Thank you to Universitas Negeri Surabaya for providing the infrastructure so that this research can be carried out. Thank you to Fitria for helping with the acquisition of research data

References

[1] Majt´an J., B´ılikov´a, K., Markovi˘c O., J´an Gr´of, Kogan G., and Sim´uth. : J. *Intern. J. Biol. Macromol*,. 2007, 40, 237–241
[2] Chen, J.K, Shen,C.R.,Liu, C.L. : *Marine. Drugs*, 2010,8, 2493-2516
[3] Widyastuti, N. : Berita Biologi, 2007, 8(6), 547-553 http://e-journal.biologi.lipi. go.id/index.php/berita_biologi/article/view/839/607
[4] Sashiwa H, Fujishima S, Yamano N, Kawasaki N, Nakayama A, Muraki E, Aiba S. *Chem. Lett.* 2001;31:308–309
[5] Ilankovan P., Hein S., Chuen-How Ng, Trung T.S., Stevens W.F. : *J.Carb.Poly.*, 2005, available online at www.sciencedirect.com
[6] Chang K.L.B and FU, W.R. :*J. of Food and Drug Anal.*,2000, 8(2) : 75-83
[7] Krokeide, I.M., Eijsink, V.G.H., and Sørlie, M. *Thermochimica Acta*., 2007, 454: 144–146
[8] Jamialahmadi,K., J.Behravan, MF.Najafi, MT.Yazdi, AR. Shahverdi, and MA.Faramarzi, *Biotechnology*.. 2011, 10(3) : 292-297.
[9] Coutin˜o, L.R., Marı´a del Carmen, M.C., Huerta, S., Revah, S., Shirai, K., *Process Biochemistry*, 2006, 41 : 1106–1110.
[10] Donzelli P.B., Ostroff G., Harman G.E., *Carbohydrat*. 2003, 338 (18) : 1823 – 1833
[11] Kumar, C., Parrack, P., W. J. of Microb. & Biotech., 2003, 19(3), 243–246
[12] Kumari M, Sharma A, and Jagannadham. : . *J. Agric. Food Chern.*, 2010.
[13] Widhyastuti,N. LIPI. 2010
[14] Ryosuke, K., Yoshiharu, M., Kazuaki, K. Kazuo, S. : 2002. *JP Patent NO. 200281696*
[15] Zhan, W.S., 2007.*CN Patent NO. 1907993*
[16] Seo, S.; King, J.M.; Prinyawiwatkul, W. : *J. Food Sci.*, 2007, *72*, C522–C526
[17] Pecsok R.L., Shields L.D., Cairns T., and McWilliams I.G. : *Modern methods of chemical analysis.* 1976, 2nd edition.