African J. Biol. Sci., 13 (1): 91-98 (2017)
ISSN 1687-4870 **e- ISSN 2314-5501 (online)**
www.aasd.byethost13.com **E.mail: aasdjournal@yahoo.com**

Production of N-acetyl-D-glucosamine by submerged fermentation of the American cockroach's *(Periplaneta americana)* Chitin

Mahmoud Kamal Abdul-Aziz Salama[1]*, Nevin Ahmed Ibrahim[2], Amany Soliman Khaled[1] and Magda HassanAbdul-AzizRadi[1]
1- Entomology Department, Faculty of Science, Ain-Shams University
2- Microbiology Department, Faculty of Science, Ain-Shams University
*corresponding author: Mahmoud.3ziz@sci.asu.edu.eg

ABSTRACT

N-acetyl-D-glucosamine (NAG) is a medical important molecule that linked linearly through β (1,4)– glycosidic linkages forming chitin. Chitin was isolated from the American cockroach's dried exoskeletons and characterized by Infrared spectroscopy (IR), the data revealed that it was in α form with 108% degree of acetylation (DA). The isolated chitin in the colloidal form was used in submerged fermentation for NAG production using the chitinolytic entomopathogenic fungus *Metarhizium anisopliae*, AUMC 2837 which showed a great potentiality on chitin degradation and NAG production at starting pH rang 3-5. The maximum NAG production was estimated at initial pH value 4, where the fungus could produce 12.52±0.46 g/l NAG from 15 g/l colloidal chitin (approximately 83.46 % of the chitin degraded to NAG) after 96 hours of incubation. Such result highlighted *Periplaneta Americana* chitin to be a promising alternative source for NAG production. This is the first report on bio-production of NAG from American cockroach's chitin by a native entomopathogenic fungus.

Key words: Chitin, N-acetylglucoseamine, fermentation, chitinases, chitinolytic activity, pH optimization.

INTRODUCTION

Recently, N-acetyl-D-glucosamine (NAG) and its derivatives have been utilized in dietary supplements and for therapeutic development due to its unique characteristics, since toxicity tests revealed that NAG is non-toxic and supporting the essential safety issue. NAG is the monosaccharide that linked linearly through β (1,4) – glycosidic linkages forming chitin, the second most abundant biopolymer in nature after cellulose and the main constituent of insect's exoskeletons (Chen *et al*., 2010).

Insects show great diversity between their relatives in the animal kingdom. They are untapped enormous biomass on earth and some of them are harmful pests cause a lot of economic and health problems. Until now, however, only limited numbers of insect species have been documented to be sources for chitin production (Liu *et al*., 2012; Wanule *et al*., 2014; Kaya & Baran, 2015).The American cockroach *Periplaneta americana* is one of the most familiar omnivorous insect distributed everywhere and causes many problems to public health.The body of adults ranged 35–40 mm in size. It is an opportunistic feeder and it is a scavenger consumes decaying organic matter. It shows excessive growth in hot, humid conditions and food abundancy. It can achieve huge numbers with extreme reproduction and development in moist and warm areas (Barbara, 2014; Wanule *et al*., 2014; Kaya & Baran, 2015).Thus *P. americana* may show great potentiality in being alternative source for chitin if mass

reared on food wastes (Wanule *et al*., 2014 ; Kaya & Baran, 2015).

Conventional method for extraction of chitin from arthropods exoskeleton is a multi-step chemical process depends mainly on two steps; demineralization by hot mineral acids and deproteinization by hot alkali. Also, removal of fats and pigments using organic solvents and oxidizing agents respectively can be used in some cases as additional steps to enhance the purity of the extracted chitin. The extracted chitin may exhibit three crystalline forms: α, β and gamma forms depending on the chains arrangement(Zhang *et al*., 2000; Liu *et al*., 2012;Arbia *et al.*, 2013; Kaya & Baran, 2015).Generally, NAG is produced by acid hydrolysis of chitin, a process that is mainly performed in concentrated acid at high temperature. This procedure, however, has some problems such as high cost; low yield (below 65%) and acidic wastes (Patil & Jadhav, 2014). Chitin degradation could be conducted enzymatically (via the action of purified chitinolytic enzymes) or using biotransformation methods by using the whole chitinolytic microorganism in the degradation process (Chen *et al*., 2010). Chitinolytic enzymes contain endochitinases, exochitinases, chitobiosidases and N-acetylglucos-aminidases (NAGases) (Cohen-kupiec & Chet, 1998). The Chitinolytic activity of microorganisms starts by increasing the solubility of chitin as the first step in degradation process; the endochitinases within microorganisms cleave chitin randomly generating the soluble and low molecular weight derivatives (oligomers). Then, the exochitinases or chitobiosidases in some organisms catalyze the cleavage of dimers. Finally, the produced oligomers and dimers by endochitinases and exochitinases are hydrolyzed by NAGases, to generate NAG (Cohen-kupiec & Chet, 1998; Lee *et al*.,1999).Chitinolytic enzymes are produced by wide variety of microorganisms including fungi (Chen *et al*., 2010; Inokuma *et al*., 2013). *Metarhizium anisopliae* was also reported to have chitinolytic activity(Rustiguel *et al*.,2012 ; Narendrakumar *et al*.,2015).

As chitinase has remarkable applications in various fields, large scale production is necessary. Submerged fermentation using filamentous fungi having a chitinolytic activity seems to be the low cost and prospective alternative for large-scale production of chitinases. A submerged fermentation-based production process has been employed that uses several filamentous fungi worldwide (Hsieh *et al*., 2007; Sitanggang *et al*., 2012).

Therefore, the present study was aimed to evaluate the efficiency of NAG production from the American cockroach's chitin using submerged fermentation process by a native isolate of *Metarhizium anisopliae* at different initial pH value. This is the first report on bio-production of NAG from the American cockroach's chitin by a native entomopathogenic fungus.

MATERIALS AND METHODS

1. Samples Collection and Chitin Extraction:

Samples of the American cockroach *P. americana* were collected by hand picking from different places in Cairo, Egypt. These samples were killed using chloroform and dried in the oven for 2 hours at 150 ^{o}C. The dried exoskeletons were ground and used for chitin extraction according to the procedure reported by Kaya and Baran (2015)with some modifications. 100g of the dried exoskeleton was refluxed with 1 M NaOH for 2 days at 90 ^{o}C and the extracted chitin was dried and characterized by Infrared spectroscopy(IR). According to Liu *et al*. (2012) the purity of the extracted chitin was indicated by calculating the

degree of acetylation (DA) using the formula:

DA = (A1655/A3450) × 100 (1)

2. Colloidal Chitin Preparation:

Fifteen grams from the extracted cockroaches' chitin powder was dissolved in 200 ml of concentrated HCl with stirring for 3 min at 40°C. The chitin was precipitated as a colloidal suspension by slowly adding cold water (2 L) adjusted to 5°C. Colloidal suspension was collected by filtering through coarse filter paper; then the filtered colloidal particles were washed with tap water until the pH value was about 4.0 (Kang *et al.*, 1999).

3. Microorganism and Inoculum Preparation:

3.1 Microorganism and maintenance:

A native isolate of the fungal species *Metarhizium anisopliae* AUMC 2837 purchased from the culture collection of Assiut University Mycological Center (AUMC) was used in this study. Potato dextrose agar medium (PDA: Potato decoction; 200g**,** Glucose; 20g**,** Agar; 20g**,** Chloramphenicol; 250mg**,** Distilled water; 1000ml, pH 6.2) was used for the maintenance and sub-culturing.

3.2 Inoculum preparation:

Spores suspension of *Metarhizium anisopliae* AUMC 2837 was prepared by flooding a fully sporulated culture grown at 28˚C for 7 days on PDA slants with sterilized saline solution (0.9% NaCl) and shaking vigorously for 1 min. Spores were counted by a hemocytometer and the concentration of spores was adjusted to approximately 7.8×10^6 spores/ml.

4. Chitin Degradation by *Metarhizium anisopliae* AUMC 2837:

4.1 Production medium preparation:

The medium used for production of NAG in submerged fermentation was prepared according to the method described by Inokuma *et al*. (2013) with some modifications. The total amount of the previously prepared suspension of colloidal chitin (15 g) was suspended in 1 liter distilled water and supplemented with 5 g of yeast extract, 1 g peptone, 1 g calcium chloride hydrate, 0.75 g magnesium sulfate, 7.5 g of ammonium chloride, 5 g of ammonium sulfate and 4 g potassium dihydrogen phosphate.

4.2 Determination of the optimal pH for NAG production:

The fungal isolate (*M. anisopliae* AUMC 2837) was subjected to different cultural pH values to determine the optimum initial pH value for NAG production. This parameter was chosen to optimize the process of chitin degradation because the production and activity of the fungal chitinases and, consequently, the NAG production was reported to be dramatically influenced by the pH value (Karthik *et al*., 2014). The initial pH of the production medium was adjusted to 2, 3, 4, 5, 6, 7 or 8 using 0.1 N HCl or 0.1 N NaOH. One ml of the previously counted spore suspension was inoculated on 20 ml of the sterilized production medium in a 100-ml screw tip autoclavable bottle for each pH value (3 replicates were conducted), then incubated at 28^{o}C for 96 hours with shaking at 120 rpm. After 96 hours, the medium was filtered using Whitman no. 1 filter papers and the filtrate was then used for the estimation of the produced NAG concentration according to the procedure used by Yanai *et al.* (1992). Two ml from the filtrate was centrifuged at 12000 rpm. Five hundred µl of the supernatant was mixed with 100 µl of potassium tetra borate (0.8 M boric acid, adjusted to pH 10.2 with KOH). The solution was heated in boiling water bath for 3 min. After cooling, 3 ml of

p-dimethyl aminobenzaldehyde solution (1 g of p-dimethyl aminobenzaldehyde in 100 ml of glacial acetic acid containing 1% v/v hydrochloric acid) was added and the mixture was incubated for 20 min at 37°C. Absorbance (A) at 585 nm was measured against water as a blank.

4.3 Statistical analysis:

Data was collected and analyzed statistically by IBM SPSS Statistics v. 20, software. One-way ANOVA test was conducted to determine the optimum pH value for degradation of chitin and production of NAG by *Metarhizium anisopliae* AUMC 2837.

RESULTS AND DISCUSSION

1. IR spectra of the extracted chitin:

IR Spectral peaks for the extracted chitin from the dried exoskeletons of *P. americana* were recorded at (3443, 3267, 3107, 2959, 2924, 2890, 2849, 2359, 2340, 1658, 1628, 1560, 1424, 1379, 1319, 1260, 1204, 1160, 1115, 1071, 1029, 954, 897 cm^{-1}). The degree of acetylation (DA) was calculated according to equation (1) to be 108.7% (Fig. 1**)**. This DA value of the extracted chitin indicates high purity of this extract.

The IR spectra of chitin from *P. americana* was comparable to α-chitin of *P. americana* that reported by Wanule *et al.* (2014) and Kaya & Baran, (2015). The spectra were characterized by three significant amide peaks at 1,658, 1,560 and 1,319 cm^{-1}, which corresponded to the amide I stretching of C=O, the amide II of N-H and amide III of C-N, respectively. It is realized that the amide I band of α-chitin splits at 1,658 and 1,628 cm^{-1}, which is attributed to the two types of H-bonds formed by amide groups in the antiparallel alignment present in crystalline regions of α-chitin (Focher *et al.*, 1992;Dutta *et al*., 2004).

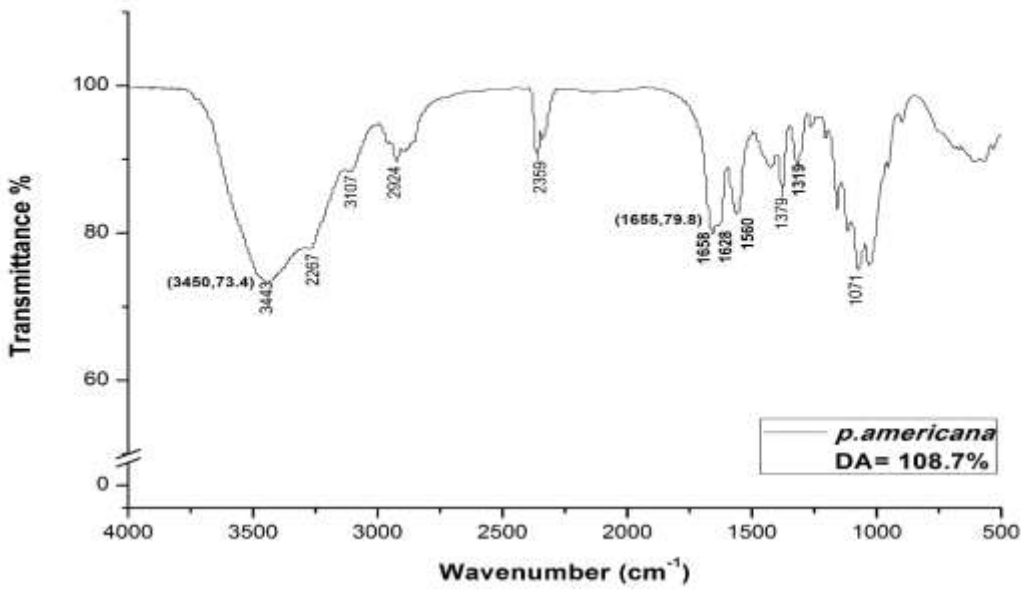

Fig. 1:The IR spectra of the chitin extracted from the American cockroach *P. americana.*

2. **NAG production by *Metarhizium anisopliae* AUMC 2837 using the extracted cockroach's chitin*:***

Metarhizium anisopliae AUMC 2837 was observed to biotransform the extracted cockroach's chitin colloidal particles directly in the production medium and NAG was released. This result affirmed the possibility of using the whole chitinolytic fungi in chitin degradation and NAG production. The same conclusion was reported by Li *et al.* (2005) who used whole bacterial strain (*Aeromonas caviae* DYU-BT4) for biotransformation of chitin and production of NAG. The statistical analysis results revealed the strong influence of initial pH of the production medium on the NAG production by *Metarhizium anisopliae* AUMC 2837. The rate of NAG production from the extracted cockroach's chitin at different initial pH values was clarified in Figure (2) & Table (1). It was generally observed that the initial pH of the medium plays an important role in the production of fungal chitinases and, consequently, the NAG production (Karthik *et al*., 2014). In the present study, NAG yield was compared in a broad range of initial pH (2–8) in shake flask cultures. It was found that *Metarhizium anisopliae* AUMC 2837 grown on medium of initial pH 4 produced maximum NAG yield (12.52±0.46 g/l). This was followed by pH value of 3, which recorded 9.81±1.54 g/l NAG and pH value 5 which measured 9.3±0.86 g/l NAG with no significant difference between them. Beyond these pH values, NAG production was found to be decreasing. This finding suggesting that the fungal chitinase responsible for chitin degradation and NAG production was stimulated by acidic pH and this result was in accordance with Massimiliano *et al*. (1998) who reported that chitinase production by *Penicillium janthinellum* was optimized at pH 4. Another study conducted by El-katatny *et al.* (2001) also showed that production of chitinase by *Trichoderma harzianum* was markedly affected by pH with the optimum at 5.5 for degradation of colloidal chitin in submerged Fermentation by *Trichoderma harzianum* (Nampoothiri *et al.*, 2004). Sharaf (2005) reported pH 5 as an optimum for chitinases production and degradation of colloidal chitin in submerged fermentation by *Alternaria alternaria*. Duo-Chuan *et al.* (2005) marked pH 6 as an optimum for chitinases production and degradation of colloidal chitin in submerged Fermentation by *Talaromyces flavus* while, Wasli *et al*. (2009) reported pH 4 as an optimum for chitinases production and degradation of colloidal chitin in submerged fermentation by *Trichoderma virens.* Agrawal & Kotasthane (2012) recorded pH 4.7 as an optimum for chitinases production and degradation of colloidal chitin in submerged fermentation by *Trichoderma aureoviride.*

Table 1*:* The NAG production by *Metarhizium anisopliae* AUMC 2837 usingthe chitin extracted from *P. americana* at different initial pH values.

pH (Value)	Corresponding letter	N (replicates)	Mean NAG(±SD) g /l	*LSD
2	A	3	2.29±0.42	AF
3	B	3	9.81±1.54	Bd
4	C	3	12.52±0.46	C
5	D	3	9.3±0.86	BD
6	E	3	3.72±0.32	EF
7	F	3	2.72±0.58	EFA
8	G	3	0.77±0.46	G

*LSD: Least significant difference at (0.05 level of significance).
-Single letter in LSD: indicates that the mean concentration of NAG produced at this initial pH value was significantly differed from that produced at other initial pH value.
-Double or Multi letters in LSD: refers to non-significant difference between mean NAG concentrations produced at the initial pH values corresponding to these letters.

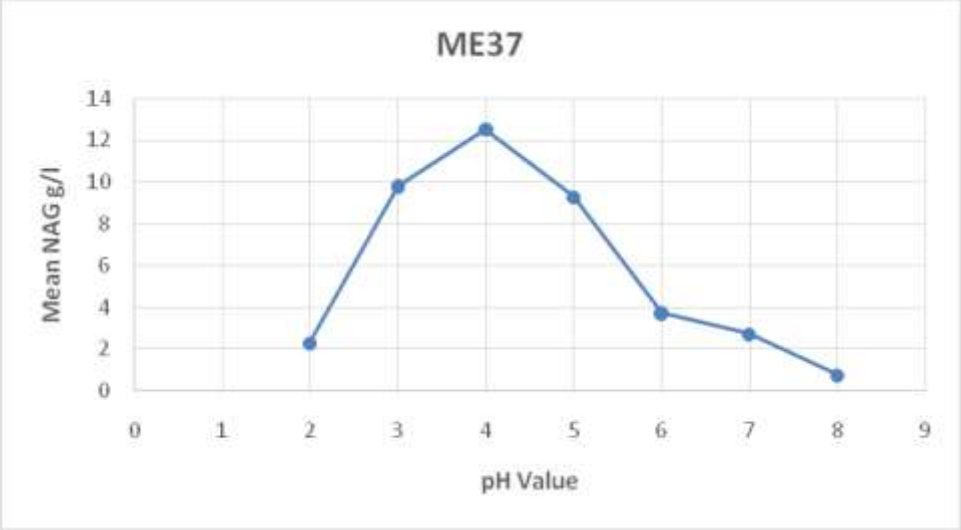

Fig. 2: The NAG production by *Metarhizium anisopliae* AUMC 2837 using extractedcockroach'schitin at different initialpH values.

Conclusion

The American cockroach *Periplaneta americana* is a cosmopolitan species that can be cultured easily. This species multiple in hot and humid conditions. Since this species is an invader and can be easily cultured, this organism could be considered as an alternative chitin source to crab, shrimp, crayfish and prawn(Kaya and Baran, 2015) , consequently for NAG.The present investigation proved that at the optimum initial pH condition, the native isolate of *Metarhizium anisopliae* (AUMC 2837) produced 12.52±0.46 g/l of NAG from 15 g/l of the extracted cockroaches' chitin (in the colloidal form) which means that 83.46 % of the prepared chitin transformed to

NAG after 96 hours of incubation. Such results demonstrated that the American cockroach (*P. americana*) is a promising alternative source for NAG production using an entomopathogenic native fungal strain as biodegrading agent.To best of our knowledge, it was the first study to discuss such issue.

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إنتاج المركب إن-أسيتيل غلوكوز أمين بواسطة عملية التخمر المغمورلكيتين الصرصور الامريكي (*بيريبلانيتا اميريكانا*)

محمود كمال عبد العزيزسلامه[1] ، نيفين احمد ابراهيم[2] ، اماني سليمان خالد[1] ، ماجده حسن عبد العزيز راضي[1]

1 - قسم علم الحشرات كليه العلوم جامعه عين شمس

2 - قسم الكيمياء الحيوى كلية العلوم جامعة عن شمس

المستخلص

هدفت الدراسه عزل الكيتين من الهيكل الخارجي للصرصور الامريكي عن طريق المعالجه بمحلول هيدروكسيد الصوديوم لازاله البروتينات الهيكليه وتنقيه ماده الكيتين، وقد تم دراسه الخواص التركيبيه للكيتين المستخلص بواسطه مطياف الاشعه تحت الحمراء وتم تعريفه ومطابقته بالكيتين المستخلص من الصرصور الامريكي في الدراسات السابقه، وقد تبين ان الكيتين المستخلص من النوع الفا-كيتين، وكانت درجه الأستله 108% ، وهذه دلاله علي نقاء الكيتين المستخلص حيث اقتربت النسبه من النسبه النظريه للكيتين كامل الاستله. وقد تم اخضاع الكيتين المستخلص لعمليه تخمر بواسطه عزله محليه معرفه من فطر *الميتاريزيم* لانتاج سكر إن-أسيتيل غلوكوز أمين ذو الاهميه الدوائيه، وتهيئه الظروف ودرجه الحموضه اللازمه لتكسير الكيتين وانتاج إن-أسيتيل غلوكوز أمين، وحددت القيمه 4 كدرجه الحموضه المثلي لانتاج مركب إن-أسيتيل غلوكوز أمين من الكيتين المستخلص من الصرصور الامريكي وقدرت الكمية المنتجة من مركب إن-أسيتيل غلوكوز أمين من استخدام 15 جرام كيتين بمقدار 0.46±12.52جرام/لتر اي ما يعادل 85.2 % من الكميه الاصليه للكيتين المحمله في وسط النمو قد تحولت الي إن-أسيتيل غلوكوز أمين. وابرزت النتائج امكانية أستخدام حشرة الصرصور الامريكي ليكون مصدرا بديلا وواعدا لانتاج إن-أسيتيل غلوكوز أمين.