J. Agrobiotech. **Vol. 9 (2)**, 2018, p. 108–118.

ISSN 1985-5133 (Press)
ISSN 2180-1983 (Online)

Isolation and Molecular Cloning of Carbohydrate Binding Module (CBM40) From *Vibrio cholerae* Non-O1 Neuraminidase

Shadariah M and Nadiawati A*

School of Agriculture Sciences and Biotechnology,
Faculty of Bioresources and Food Industry,
Universiti Sultan Zainal Abidin, Besut Campus,
22200 Besut, Terengganu, Malaysia.

Corresponding author; Nadiawati A.

School of Agriculture Sciences and Biotechnology,
Faculty of Bioresources and Food Industry,
Universiti Sultan Zainal Abidin, Besut Campus,
22200 Besut, Terengganu, Malaysia.
***Corresponding author; Email: nadiawati@unisza.edu.my**

Keywords:
Carbohydrate Binding Module (CBM)
Vibrio cholerae Non-01
Neuraminidase
Cloning

ABSTRACT

Carbohydrate binding modules (CBMs) are discrete contiguous amino acid sequence within a carbohydrate-active enzyme which are, non-catalytic modules that primarily exist to target parent enzyme to its substrate for efficient hydrolysis. Although many sialidase proteins have been identified from various pathogenic bacteria, only a few enzymes are commercially available which have been used for chemoenzymatic syntheses and therapeutics application. In order to study family 40 CBM domain, a number of bacteria have been screened including *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 14579*, Staphylococcus aureus* ATCC 33862*, Salmonella thypii* ATCC 14028 and *Vibrio cholerae* Non-O1. A gene encoding CBM40 domain was screened from all the bacteria strains and subjected to PCR amplification. From all the samples tested, only *Vibrio cholerae* Non-O1 amplified a PCR product with approximate size of 530 bp. From BLAST sequence analysis, result has shown 99% similarity with the target *Vibrio cholerae* neuraminidase, NanH (M83562). Next, the confirmed CBM40 gene was further ligated into pGEMT Easy Vector system and transformed into *E. coli* JM109 host to secure the clone before re-ligated into suitable expression vector.

Keywords: Carbohydrate Binding Module (CBM), *Vibrio cholerae* Non-01*,* Neuraminidase, Cloning

ABSTRAK

Domain perlekatan karbohidrat (CBM) adalah merupakan turutan asid amino yang berada pada enzim aktif karbohidrat yang merupakan modul bukan katalitik yang wujud untuk mensasarkan substrat kepada enzim induk untuk tujuan hidrolisis. Walaupun terdapat protein sialidase yang telah dikenalpasti dari pelbagai bakteria patogen, hanya beberapa jenis enzim sahaja yang telah dikomersialkan dan telah digunakan untuk sintesis kemoenzimatik dan aplikasi terapeutik. Dalam usaha untuk mengkaji keluarga 40 CBM, saringan telah dibuat terhadap beberapa jenis bakteria seperti *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 33862, *Salmonella thypii* ATCC 14028 dan *Vibrio cholerae* Non-O1. Kehadiran domain CBM40 telah disaring dari semua jenis bakteria tersebut melalui teknik PCR. Daripada semua sampel yang diuji, hanya sampel dari *Vibrio cholerae* Non-O1 menunjukkan hasil amplifikasi PCR dengan anggaran saiz 530 bp. Dari analisis urutan gen melalui BLAST, hasilnya menunjukkan 99% kesamaan dengan *Vibrio cholerae* neuraminidase, NanH (M83562). Selepas itu, gen CBM40 yang telah disahkan akan di sambungkan ke vektor yang dikenali sebagai pGEMT Easy Vector dan menggunakan *E. coli* JM109 sebagai host sebelum menggunakan vektor ekpresi lain yang lebih sesuai.

Kata Kunci: Domain perlekatan karbohidrat (CBM), *Vibrio cholerae* Non-01*,* Neuraminidase, pengklonan

INTRODUCTION

Carbohydrate Binding Module (CBM) is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate binding activity. Currently, it is separated into 83 defined families based on CAZy website. It can be found in any proteins domain that recognize polysaccharides such as cellulose, chitin, β-glucans, starch, glycogen, inulin, pullulan, xylan, and other different sugars such as arabinofuranose, mannan, fucose, lactose, galactose, polygalacturonic acid, β-D-galactosyl-1,4-β-D-N-acetylglucosamine, lipopolysaccharides, blood group A/B antigens (Guillén *et al*., 2009). CBMs can be found to be localized at the N- or C-terminal end of these proteins and between two catalytic modules (Abe *et al.,* 2004; Tabuchi *et al.,* 2010). Besides, CBM domain from alpha, beta-amylase from *Paenibacillus polymyxa* was found as a single unit of CBM or arranged in tandem (Kawazu *et al*., 1987). These domains could be identical among each other or very homologous (Mehta *et al*., 2016), as reported by Cameron *et al.* (2012) for *Lactobacilli* alpha-amylases; but in some cases, tandem CBMs can include modules with different substrate specificity.

The main role of CBMs is to recognize and bind specifically to carbohydrates and serve to bring the substrate to the active site for hydrolysis (Várnai *et al.*, 2013; Duan *et al.*, 2015). Some of the glycoside hydrolases possess CBMs that help target these enzymes to appropriate substrates and increase their catalytic efficiency (Herve *et al.*, 2010). In addition, the interaction between CBM and its substrate may result in different functions, such as enhanced hydrolysis of insoluble substrates, bringing the catalytic domain in close proximity to the substrate, polysaccharide structure disruption and cell surface protein anchoring (Guillén *et al.*, 2009; Herve *et al.*, 2010).

Escherichia coli (gram-negative gamma proteobacterium) is presently the best-understood organism which is use as a key tool in many genetic manipulations. Molecular cloning into one of many *E. coli* vector and host such as JM109 is a standard procedure in most molecular biology and biotechnology laboratories (Chronan, 2007). *E. coli* is also known as the workhorse of recombinant protein production since conditions have been found that allow proteins from across biology to be efficiently expressed in and purified from *E. coli* (Idalia, 2017). The *E. coli* JM109 strain is also a useful host for transformation of pGEM Vectors and for production of single-stranded DNA and blue/white screening.

In this study, screening and isolation of carbohydrate binding module (CBM) from bacteria was carried out using basic molecular biology approach. Moreover, TA cloning strategy was carried out using a suitable vector and transformed in *E. coli* host. This approach was carried out before hand in order to secure the gene of interest before conducting more studies on its protein expression, purification and characterization. Although many sialidase proteins have been identified from various pathogenic bacteria, only a few enzymes are commercially available due to the limited enzyme availability and low synthetic yield. Moreover, their therapeutics potential of this CBMs are not quite yet been explored.

MATERIAL AND METHODS

Genomic DNA Extraction

An overnight bacteria cultures of *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 14579*, Staphylococcus aureus* ATCC 33862*, Salmonella thypii* ATCC 14028 and *Vibrio cholerae* Non-O1 were subjected to DNA extraction using Wizard genomic DNA purification kit (Promega). The cultures were centrifuged 13000-16000 x g for 2 min to obtain pellet. The nuclei lysis solution was added to lyse the cells by gently pipetting and then incubated for 5 min at 80 ºC. About 3 µl of RNase solution was added and incubated at 37 ºC for 15-60 min to remove RNA. In order to remove protein in the precipitate, about 200 µl of protein precipitation solution was added and vortex few times before incubated on ice for 5 min. The samples were then centrifuged at 13000-16000 x g for 3 min. The supernatant was transferred into tube containing 600 µl of room temperature isopropanol and proceed to centrifugation. The supernatant was decanted. About 600 µl of room temperature 70% ethanol was added and mixed before centrifuged at 13000-16000 x g for 2 min. The ethanol was aspirated and pellet was air-dried for 10-15 min. Lastly, the DNA pellet was rehydrated in 50 µl Rehydration solution for 1 h at 65 ºC or overnight at 4 ºC. Samples concentration were calculated using Biophotometer and the ratio of A_{260}/A_{280} was used to indicate the purity of nucleic acid.

DNA Electrophoresis

DNA samples were separated by gel agarose electrophoresis method adapted from Sambrook (1989) with a slight modification. About 1% agarose gel and 1X GelRed was added as a staining agent. The electrophoresis was conducted at 80 Votts, 500 mA for 60 min. Next, the gel was visualized under the gel documentation system (Luminescent Image Analyzer) to view and analysed the genomic DNA fragments from all samples.

PCR amplification optimization of CBM40 domain

Gradient PCR amplification was carried out in a final volume of 25 µl reaction mixtures containing 1X buffer PCR, 2 mM of $MgCl_2$, 10 mM dNTP, 1.25 U *Taq* DNA Polymerase (Promega), 300 ng template DNA, 1 µM of primers (Table 1). About 35 cycles of amplification reactions were conducted according to the following parameters 95°C for 2 minutes (pre-denaturation), 95°C for 1 minute (denaturation), 50-58°C for 1 minute (annealing), 72°C for 5 minutes (extension) and 72°C for 5 minutes (final extension). Two primers were used which were 1F2 and 1R2 primer (Table 1). Successfully amplified fragments were visualized under the gel documentation system (Luminescent Image Analyzer). Next, the fragments were sent for sequencing service at 1st Base Laboratory Sdn Bhd for sequence confirmation.

Table 1. Primers for CBM40 gene amplification

Primer	Tm (°C)	GC content (%)	Sequence of primer
1F2 (F)	51.9	40.9	5'- GTC CAC TTT TTG ACT ATA ACG C-3'
1R2 (R)	58.6	48.0	5'- CGG CTA GTC GCC TTG AAT TTC AAA C-3'

Preparation of competence cell

The following procedure is a modified method of Cohen *et al.* (1972) for the preparation of *E. coli* competent cells. About 10 ml of LB broth was inoculated with a single colony of *E. coli* JM109 and incubated overnight at 37 °C, 150 rpm. Next, 0.4 ml of the overnight culture was then inoculated into 40 ml (1:100 dilutions) LB broth and incubated at 37 °C with vigorous shaking until A_{600} reached 0.5. Then, the cells were centrifuged at 6000 rpm for 5 min at 4 °C. The supernatant was removed and about half of the original volume of 100 mM $CaCl_2$ was added and the pellet was resuspended gently. This mixture was then incubated on ice for 20 min followed by centrifugation for 5 min at 6000 rpm at 4 °C. The supernatant was decanted and the pellet was resuspended in 0.1 of original volume of 100 mM $CaCl_2$. Glycerol was added to a final concentration of 20 % (v/v). The competence cells were then aliquoted into 200 µl volume in sterile tubes and kept at -80 °C until used.

Ligation of DNA

Using the pGEM-T Easy Vector System (Promega), the PCR products were directly cloned into the vector without being digested. The ratio of vector: insert of 1:3 was used. About 50 ng of pGEM-T Easy vector was mixed with 1 µg of PCR products,1X T4 DNA ligase buffer and 0.3 U of T4 DNA ligase. Sterile dH_2O was added up to 10 µl of the final volume and the mixture was incubated overnight at 4 °C. The next day, the mixture was briefly centrifuged and used to transform competent cells.

Transformation into *E. Coli* JM109 by heat shock method

The frozen competent cells were thawed to room temperature and immediately placed on ice. About 5 µl of the ligation reaction was added to 200 µl of competent cells. The mixture was gently flicked and incubated on ice for 20 min. The cells were then heat-shocked at 42 °C for 45 sec and returned to the ice bath for another 2 min. Then, 800 µl of LB broth was added to the mixture and incubated at 37 °C for 90 min with shaking at 150 rpm. The mixture was centrifuged at 8000 rpm at room temperature and about 800 µl of the supernatant was removed

out. About 100 µl of each transformation was plated onto LB plates containing 100 µg/ml of ampicillin. The plates were incubated overnight at 37 °C.

Confirmation of insert by colony PCR

Colonies containing recombinant vector DNA were screened using colony PCR amplification. Several colonies were randomly picked by sterile toothpicks and each was resuspended in 10 µl of distilled water and was heated at 99 °C for 5 minutes before cell debris was removed by centrifugation for 1 minute. An aliquot of 10 µl of the supernatant was transferred to a fresh 0.5 ml tube for PCR. The tube was stored on ice before used. PCR amplification was carried out based on protocol mentioned earlier. Colony with the presence of the correct insert size was subjected to sequencing service at 1st Base Laboratory Sdn Bhd.

Sequence Aligment

Complete gene sequence derived from the sequencing analysis was aligned with several reference bacterial neuraminidases (Accession number: APF83226, 2W68_A, CSB55961 and M83562) based on BLAST analysis result. For the sequence aligment analysis, Clustal Omega software was used.

Statistical analysis

Experimental data were analyzed using Statistical data analysis for Excel. The reported results in the present study were represented as the mean values of triplicates ± the standard deviation.

RESULTS AND DISCUSSION

DNA extracted from all five bacteria strains were shown in Figure 1 and the purity of all DNA samples were analysed at A_{260}/A_{280} with all the samples were within the range of good quality DNA 1.7–2.0. In order to amplify CBM40 region, gradient PCR amplifications optimization were carried out using temperature range of 50 ºC to 58 ºC as showed in Figure 2. It shows no amplified product at temperature 50ºC to 58ºC for *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 14579*, Staphylococcus aureus* ATCC 33862 and *Salmonella thypii* ATCC 14028. However, Figure 3 shows PCR products with approximate size of 530 bp was successfully amplified at all annealing temperature from *Vibrio cholerae* Non-O1. Sample duplication was done to further confirm the results. The fragment was analysed by DNA sequencing and analysed by BLAST software. The DNA fragment was identified encoding for Family 40 carbohydrate binding module (CBM) was then proceed to ligate into pGEM-T Easy vector and were transformed into *E. coli* JM109 using heat shocked method.

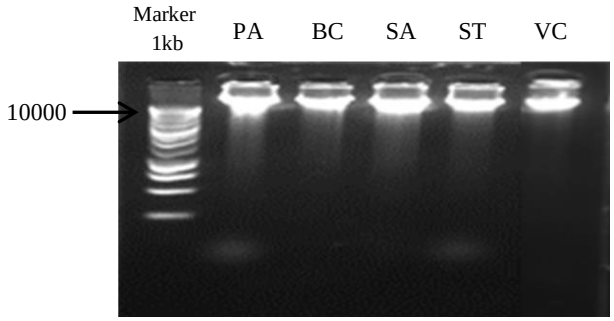

Figure 1. DNA extraction from all bacteria; *Pseudomonas aeruginosa* ATCC 27853 (PA), *Bacillus cereus* ATCC 14579 (BC), *Staphylococcus aureus* ATCC 33862 (SA), *Salmonella thypii* ATCC 14028 (ST) and *Vibrio cholerae* Non-O1 (VC).

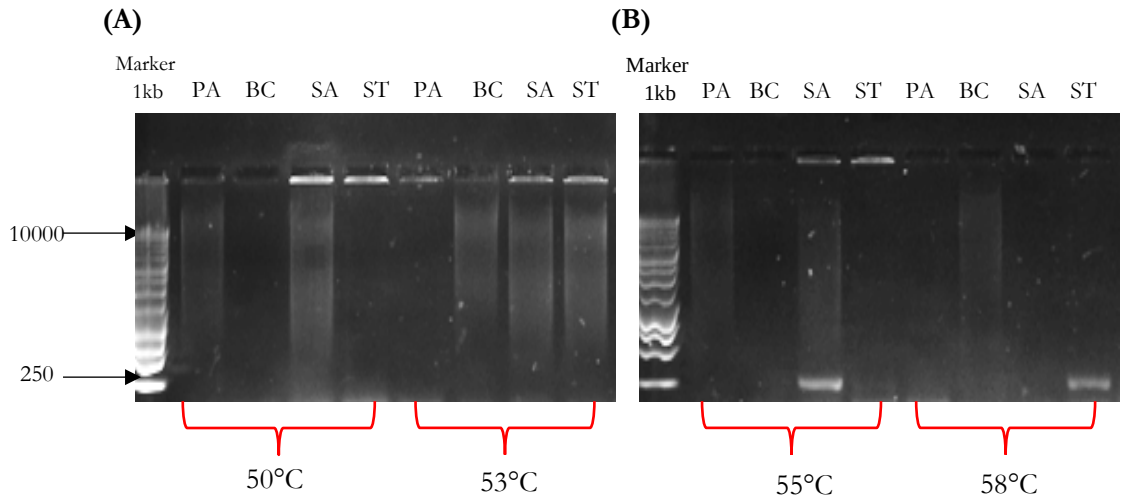

Figure 2. Gradient PCR amplification at (A) 50ºC and 53ºC and (B) at 55ºC and 58ºC. *Pseudomonas aeruginosa* ATCC 27853 (PA), *Bacillus cereus* ATCC 14579 (BC), *Staphylococcus aureus* ATCC 33862 (SA) and *Salmonella thypii* ATCC 14028 (ST).

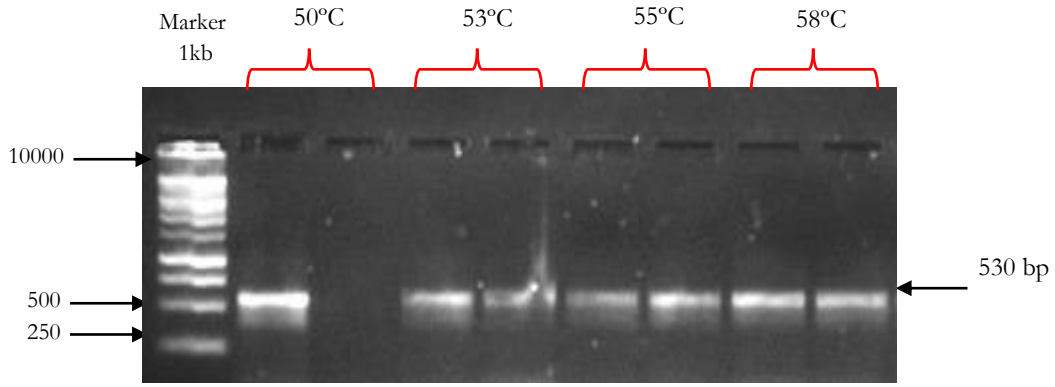

Figure 3. Gradient PCR amplification of V. *cholerae* Non-O1 at annealing temperature of 50, 53 ºC, 55 ºC.

pGEM-T Vector is one of the vector with T-overhang at both ends which is suitable for TA cloning strategy. TA cloning is a popular method of cloning without the use of restriction enzymes. Instead, PCR products are amplified with only Taq DNA polymerase and other polymerases (Zhou *et al.*, 2000). These polymerases lack 5'-3' proofreading activity and add an adenosine triphosphate residue to the 3' ends of the double-stranded PCR products. Such PCR amplified products can thus be cloned in a linearized vector that has complementary 3' thymidine triphosphate overhangs. The major problem is that, the gene has a 50% chance of getting cloned in the reverse direction (Zuo and Rabie, 2010).

For further confirmation of the insert, colony PCR was carried out on the putative clones to re-confirm the presence of CBM40 (Figure 4). Colony PCR is a high-throughput and reliable method used to detect a recombinant insert using the DNA within the transformed cells as a template (Walch *et al.*, 2016). In brief, a sterile pipet tip is used to collect a small amount of cells. These cells are heated to 94°C to release the plasmid DNA from the cell which served as template for the amplification reaction. Primers usually designed to specifically target the insert DNA or alternatively, targeting vector DNA flanking the insert (Walch *et al.*, 2016). In this study, primer 1F2 and 1R2 were designed based on the conserved region flanking the N-terminal and C-terminal domain of carbohydrate binding domain from bacteria neuraminidases from the NCBI database.

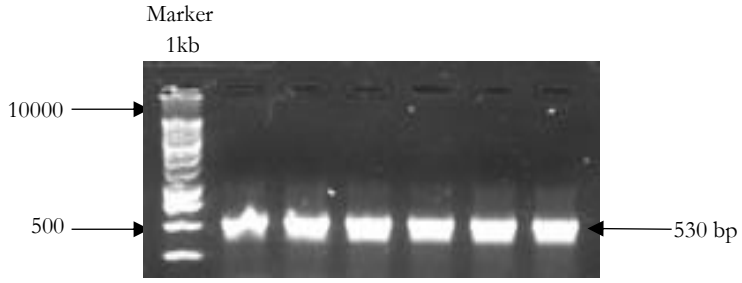

Figure 4. Colony PCR perfomed on the putative clones carrying gene of interest with amplification product approximate size of 530 bp.

```
APF83226        MRFKNVKKTALMLAMFGMATSSNAALFDYNATGDTEFDSPAKQGWMQDNTNNGSGVLTNA      60
2W68_A          ---------------------AAMALFDYNATGDTEFDSPAKQGWMQDNTNNGSGVLTNA      39
CSB55961        MRFKNVKKTALMLAMFGMATSSNAALFDYNATGDTEFDSPAKQGWMQDNTNNGSGVLTNA      60
PA01            ----------------------------------------QGWMQDNTNNGSGVLTNA      18
M83562          MRFKNVKKTALMLAMFGMATSSNAALFDYNATGDTEFDSPAKQGWMQDNTNNGSGVLTNA      60
                                                         ******************

APF83226        DGMPAWLVQGIGGRAQWTYSLSTNQHAQASSFGWRMTTEMKVLSGGMITNYYANGTQRVL      120
2W68_A          DGMPAWLVQGIGGRAQWTYSLSTNQHAQASSFGWRMTTEMKVLSGGMITNYYANGTQRVL      99
CSB55961        DGMPAWLVQGIGGRAQWTYSLSTNQHAQASSFGWRMTTEMKVLSGGMITNYYANGTQRVL      120
PA01            DGMPAWLVQGIGGRAQWTYSLSTNQHAQASSFGWRMTTEMKVLSGGMITNYYANGTQRVL      78
M83562          DGMPAWLVQGIGGRAQWTYSLSTNQHAQASSFGWRMTTEMKVLSGGMITNYYANGTQRVL      120
                ************************************************************

APF83226        PIISLDSSGNLVVEFEGQTGRTVLATGTAATEYHKFELVFLPGSNPSASFYFDGKLIRDN      180
2W68_A          PIISLDSSGNLVVEFEGQTGRTVLATGTAATEYHKFELVFLPGSNPSASFYFDGKLIRDN      159
CSB55961        PIISLDSSGNLVVEFEGQTGRTVLATGTAATEYHKFELVFLPGSNPSASFYFDGKLIRDN      180
PA01            PIISLDSSGNLVVEFEGQTGRTVLATGTAATEYHKFELVFLPGSNPSASFYFDGKLIRDN      138
M83562          PIISLDSSGNLVVEFEGQTGRTVLATGTAATEYHKFELVFLPGSNPSASFYFDGKLIRDN      180
                ************************************************************

APF83226        IQPTASKQNMIVWGNGSSNTDGVAAYRDIKFEIQGDVIFRGPDRIPSIVASSVTPGVVTA      240
2W68_A          IQPTASKQNMIVWGNGSSNTDGVAAYRDIKFEIQGD------------------------      195
CSB55961        IQPTASKQNMIVWGNGSSNTDGVAAYRDIKFEIQGDVIFRGPDRIPSIVASSVTPGVVTA      240
PA01            IQPTASKQNMIVWGNGSSNTDGVAAYRDIKFEIQGD------------------------      174
M83562          IQPTASKQNMIVWGNGSSNTDGVAAYRDIKFEIQGDVIFRGPDRIPSIVASSVTPGVVTA      240
                ***********************************
```

Figure 5. Amino acid sequence alignment of Carbohydrate Binding Module (CBM40) from *Vibrio cholerae* Non-O1 with bacterial Neuraminidases with Accession No. APF83226, 2W68_A, CSB55961 and M83562 by Clustal Omega software.

```
  1 CAG GGC TGG ATG CAG GAC AAC ACC AAC AAC GGC AGC GGC GTG CTG ACC AAC GCC GAC GGC  60
  1  Q   G   W   M   Q   D   N   T   N   N   G   S   G   V   L   T   N   A   D   G  20

 61 ATG CCC GCC TGG CTG GTG CAG GGC ATC GGC GGC AGG GCC CAG TGG ACC TAC AGC CTG AGC 120
 21  M   P   A   W   L   V   Q   G   I   G   G   R   A   Q   W   T   Y   S   L   S  40

121 ACC AAC CAG CAC GCC CAG GCC AGC AGC TTC GGC TGG AGG ATG ACC ACC GAG ATG AAG GTG 180
 41  T   N   Q   H   A   Q   A   S   S   F   G   W   R   M   T   T   E   M   K   V  60

181 CTG AGC GGC GGC ATG ATC ACC AAC TAC TAC GCC AAC GGC ACC CAG AGG GTG CTG CCC ATC 240
 61  L   S   G   G   M   I   T   N   Y   Y   A   N   G   T   Q   R   V   L   P   I  80

241 ATC AGC CTG GAC AGC AGC GGC AAC CTG GTG GTG GAG TTC GAG GGC CAG ACC GGC AGG ACC 300
 82  I   S   L   D   S   S   G   N   L   V   V   E   F   E   G   Q   T   G   R   T 100

301 GTG CTG GCC ACC GGC ACC GCC GCC ACC GAG TAC CAC AAG TTC GAG CTG GTG TTC CTG CCC 360
101  V   L   A   T   G   T   A   A   T   E   Y   H   K   F   E   L   V   F   L   P 120

361 GGC AGC AAC CCC AGC GCC AGC TTC TAC TTC GAC GGC AAG CTG ATC AGG GAC AAC ATC CAG 420
121  G   S   N   P   S   A   S   F   Y   F   D   G   K   L   I   R   D   N   I   Q 140

421 CCC ACC GCC AGC AAG CAG AAC ATG ATC GTG TGG GGC AAC GGC AGC AGC AAC ACC GAC GGC 480
141  P   T   A   S   K   Q   N   M   I   V   W   G   N   G   S   S   N   T   D   G 160

481 GTG GCC GCC TAC AGG GAC ATC AAG TTC GAG ATC CAG GGC GAC 522
161  V   A   A   Y   R   D   I   K   F   E   I   Q   G   D 174
```

Figure 6. Carbohydrate Binding Module; Family 40 (CBM40) sequence from *Vibrio cholerae* Non-O1. Deduced amino acid sequence is shown in one-letter code under the DNA sequences.

The amplified fragment with approximate size of 530 bp was sent out for sequencing service and the sequences were analysed using BLASTN and BLASTX softwares from NCBI database. The result showed 99% similarity to *Vibrio cholerae* Neuraminidase with Accession No. APF83226, 2W68_A, CSB55961 and M83562 (Figure 5). The analysis and comparisons were performed with EMBOSS WATER, CLUSTALW, SEQUENCE MANIPULATION SUITE (SMS), MUSCLE and BLAST programs. After analyses were done on the sequence, the deduced amino acid sequence of CBM 40 domain from *Vibrio cholerae* Non-O1 consist about 174 amino acid (522 bp) as shown in Figure 6.

Bacteria neuraminidase/sialidase can contain accessory modules attached to the catalytic core of the protein. For example, the *NanH* sialidase from *Vibrio cholerae* is known to be composed of a canonical six-bladed β-propeller catalytic domain that has two CBMs that flank the catalytic domain (Crennell *et al.,* 1994). Both CBM domains share the same structure topology despite sharing only 23% sequence identity (Moustafa *et al.,* 2004). Other bacteria, for example *Clostridium perfringens* NanJ sialidase also possess the same domain but its displayed lower affinity towards sialic acid as compared to *V. cholerae* CBM40 domain (Boraston *et al.,* 2007). Besides that, several other bacteria, including *Arthrobacter nicotianae*, *Arthrobacter ureafaciens*, *Clostridium perfringens*, *Pasteurella multocida* and *Streptococcus pneumoniae* produce more than one neuraminidase as isoenzymes with different biochemical features. Although, some of the biological functions of these isoenzymes are still unclear, their substrate specificities and expression patterns have been studied and display important roles in the interaction with other organisms or in the infection of a specific tissue (Manco *et al.,* 2006; Uchiyama *et al.,* 2009).

CONCLUSIONS

We have successfully isolated Family 40 Carbohydrate Binding Module (CBM) from *Vibrio cholerae* Non-O1 strain. The CBM40 gene isolated has been confirmed by sequencing service and possesed 99% similarity to the *Vibrio cholerae* neuraminidase as reported on the NCBI database. The recombinant construct was kept as glycerol stock for future protein expression and purification study. Although many sialidase proteins have been identified from various pathogenic bacteria, only a few commercially available enzymes have been used for the chemoenzymatic syntheses of sialoglycoconjugates and for therapeutics approach.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Assoc. Prof. Dr. John Tang Yew Huat from Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, Terengganu, Malaysia for supplying *Vibrio cholerae* Non-O1 strain used in this study. This work was supported by Research Acculturation Grant Scheme (RAGS)- RAGS/1/2014/SG05/UNISZA//2 from Ministry of Higher Education Malaysia.

REFERENCES

Abe, A., Tonozuka, T., Sakano, Y., Kamitori, S. (2004) Complex structures of *Thermoactinomyces vulgaris* R-47 alpha-amylase 1 with malto-oligosaccharides demonstrate the role of domain N acting as a starch-binding domain. *Journal of Molecular Biology* ***335***: 811–822.

Boraston, A. B., Ficko-Blean, E., and Healey, M. (2007). Carbohydrate recognition by a large sialidase toxin from *Clostridium perfringens*. *Biochemistry* ***46***: 11352-11360.

Cameron, E. A., Maynard, M. A., Smith, C. J., Smith, T. J., Koropatkin, N. M., and Martens, E. C. (2012). Multi-domain carbohydrate-binding proteins involved in *Bacteroides thetaiotaomicron* starch metabolism. *Journal of Biological Chemistry* ***287:*** 34614-34625.

Chandra, P. K. and Wikel, S. K. (2005). Analyzing ligation mixtures using a PCR based method. *Biological Procedures Online* ***7***: 93-100.

Chronan, J. (2014). *Escherichia coli as an experimental organism.* Encyclopedia of Life Sciences, Wiley-Blackwell.

Cohen SN, Chang AC, Hsu L (1972). Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proceeding of National Academy of Sciences of USA* ***69***: 2110-2114.

Crennell, S., Garman, E., Laver, G., Vimr, E., and Taylor, G. (1994). Crystal structure of *Vibrio cholerae* neuraminidase reveals dual lectin-like domains in addition to the catalytic domain. *Structure* ***2***: 535-544.

Duan, J., Feng, Y. L., Cao, Q. L., Huang, M. Y. and Feng, J. X. (2015). Identification of a novel family of carbohydrate-binding modules with broad ligand specificity. *Nature* ***6***: 1-8.

Guillén, D., Sánchez, S., and Rodriguez-Sanoja, R. (2009). Carbohydrate-binding domains: Multiplicity of biological roles. *Applied Microbiology and Biotechnology* ***85(5)***: 1241-1249.

Herve, C., Rogowski, A, Blake, A. W., Marcus, S. E., Gilbert, H. J. and Knox, J. P. (2010). Carbohydrate-binding modules promote the enzymatic deconstruction of intact plant cell walls by targeting and proximity effects. *Proceeding of National Academy of Sciences of USA* ***107***:15293–15298.

Idalia, V. M. N. and Bernardo, F. (2017). *Escherichia coli* as a Model Organism and Its Application in Biotechnology. *Intech Open*: 253-273.

Manco, S., Hernon, F., Yesilkaya, H., Paton, J.C., Andrew, P.W., and Kadioglu, A. (2006). Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis. *Infection and immunity* ***74***: 4014-4020.

Mehta, D. and Satyanarayana, T. (2016). Bacterial and Archaeal α-Amylases: Diversity and Amelioration of the Desirable Characteristics for Industrial Applications. *Frontiers Microbiology* ***7***: 1-21.

Mezei, L. M. and Storts, D. R. (1994). *Purification of PCR products. In: PCR Technology: Current Innovations*, Griffin, H. G. and Griffin, A. M., eds., CRC Press, Boca Raton, FL, 21 pp.

Moustafa, I., Connaris, H., Taylor, M., Zaitsev, V., Wilson, J.C., Kiefel, M.J., von Itzstein, M., and Taylor, G. (2004). Sialic acid recognition by Vibrio cholerae neuraminidase. *The Journal of biological chemistry* ***279***: 40819-40826.

Kawazu, T, Nakanishi, Y, Uozumi, N., Sasaki, T, Yamagata, H., Tsukagoshi, N., Udaka, S. (1987). Cloning and nucleotide sequence of the gene coding for enzymatically active fragments of the *Bacillus polymyxa* beta-amylase. *Journal of Bacteriology* ***169(4)*** : 1564–70.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular cloning: A laboratory manual.* Maniatis. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Tabuchi, S., Ito, J., Adachi, T., Ishida, H., Hata, Y., Okazaki, F., Tanaka, T., Ogino, C. And Kondo, A. (2010). Display of both N- and C-terminal target fusion proteins on the Aspergillus oryzae cell surface using a chitin-binding module. *Applied Microbiology and Biotechnology* ***87***: 1783-1789.

Uchiyama, S., Carlin, A.F., Khosravi, A., Weiman, S., Banerjee, A., Quach, D., Hightower, G., Mitchell, T.J., Doran, K.S., and Nizet, V. (2009). The surface- anchored NanA protein promotes pneumococcal brain endothelial cell invasion. *The Journal of experimental medicine* ***206***: 1845-1852.

Várnai, A., Siika-aho, M. And Viikari, L. (2013). Carbohydrate-binding modules (CBMs) revisited: reduced amount of water counterbalances the need for CBMs. *Biotechnology for Biofuels* ***6(30)*** : 2-11.

Walch, G., Knapp, M., Rainer, G. and Peintner, U. (2016). Colony-PCR Is a Rapid Method for DNA Amplification of Hyphomycetes. *Journal of Fungi (Basel)* ***2(2)*** : 1-12.

Zhou, M.Y. and Gomez-Sanchez, C.E.(2000). Universal TA Cloning. *Current Issues in Molecular Biology* ***2(1)*** : 1-7.

Zuo, P., Rabie, B. M. (2010). One-step DNA fragment assembly and circularization for gene cloning. *Current Issues in Molecular Biology* ***12*** : 11–16.