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Evaluation of chitinolytic enzymes activities during microbial biotransformation of *Archachatina marginata* exoskeleton to chitooligosaccharide

Simiat O. Jimoh[1*]; Khadijat O. Adefaye[1]; Lateefah A. Arowolo[2]; Ramot B. Badmos-Oladapo[1]

[1]Department of Biological Sciences (Microbiology Programme), Fountain University, Osogbo, Nigeria; [2]Department of Medicine and Surgery, Faculty of Clinical Sciences, Lagos State University, Nigeria

[*]Corresponding author E-mail: olanike771@gmail.com

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Abstract

Natural macromolecules such as polysaccharides are bioactive substances obtained from the agricultural feed stocks and\ or crustacean shell wastes, thus chitin biomass as a rich renewable resource; is an additional abundant natural polysaccharide in the environment. Despite the recent interests in the biomedical applications of chitin, complexity of chitosan and their derivatives in terms of controlling their chemical synthesis due to the formation of secondary compounds is a major problem, thus this research aimed to focus on the microbial biosynthesis of chitinous products and their derivatives from the seafood wastes. In the current research work; chitin, chitosan and chitooligosaccharides (COS) were biosynthesized from *Archachatina marginata* exoskeletons; through chemical deacetylation and microbial transformation techniques. During this study, *Archachatina marginata* exoskeleton was analyzed according to the method adopted by the Association of Official Analytical Chemists. In addition, a synthetic route for chitooligosaccharides production was investigated using a previously characterized *Saccharomyces cerevisiae* KR13 strain. The crude extract obtained after the biotransformation period was subjected to derivatization, and then the chitooligosaccharides yield, enzymes activities and their molecular weights were determined using Gas chromatography (GC). Moreover, the size exclusion chromatography revealed the presence of several enzymes, including chitin deacetylase; chitooligosaccharide deacetylase, chitinase and chitosanase; in addition to chitooligosaccharides derivatives such as; chitohexose, chitopentose, chitotetrose, chitotriose, chitobiose and chitosan with varying concentrations. Based on the results obtained in this study, manipulation of *S. cerevisiae* KR13 strain clarified the poor solubility of chitin and chitosan, which makes a challenge in using them in the biomedical applications, despite their important functional activities.

Keywords: Chitooligosaccharide, Chitin deacetylase, Chitosanase, *Saccharomyces cerevisiae*

1. Introduction

Chitooligosaccharides are byproducts of chitin or chitosan obtained through enzymatic hydrolysis using chitinolytic enzymes produced by various fungi such as; *Aspergillus fumigatus*, *Aspergillus oryzae* and *Saccharomyces cerevisiae* (Goughenour *et al*., 2021); through chemical hydrolysis using several acids (i.e. HNO_2, HCl, HF and H_3PO_4,) (Kaczmarek *et al*., 2019), or through oxidation-reduction methods using H_2O_2 (Hai *et al*., 2019). Chitooligosaccharides are hydrophilic due to their lower viscosity; petite chain length and free amino side chains in the D-glucosamine unit, thus they are being used in several biomedical applications (Palanivel *et al*., 2018). Despite of these characteristics; control of the chemical reactions is complex, due to the formation of secondary compounds that are difficult to extract (Ahn *et al*., 2021).

Chitooligosaccharides are used in several pharmaceutical and medicinal applications, due to being non-toxic; have high solubility and positive physiological effects (Liang *et al*., 2018). The beneficial biological effects of chitooligosaccharides include; decreasing blood cholesterol, decreasing excessive blood pressure, protecting against infections, controlling arthritis, enhancing calcium absorption uptake and improving the antitumor properties (Liaqat and Eltem, 2018). The cationic properties exerted by the amino acid groups present in this polymer and its molecular weight play significant roles in its antitumor potential (Liang *et al*., 2018). Furthermore, the diversity of biological activities expressed by the chitooligosaccharides depends on their degrees of acetylation, and partly based on the existing functional groups such as the amino and carboxyl groups (Kumar *et al*., 2020). On the other hand, the chitosan and chitin, which serve as raw materials for chitooligosaccharide synthesis, also have wide range of biomedical applications including tissue engineering; drug and gene delivery, wound healing, in addition to stem cell technology (Satitsri and Muanprasat, 2020). The most significant characteristics of these chitosan and chitin biopolymers that made them ideal candidates for fabricating the polymeric tissue scaffolds are their high porosity and biodegradability (Islam *et al*., 2020); predictable degradation rate, structural integrity, non-toxicity to cells and compatibility (Balaji *et al*., 2018). However, the deprived solubility of chitin and chitosan makes their utilization more difficult in the biomedical applications, despite of their important functional potentials (Song *et al*., 2018). The objective of this study was to synthesize chitooligosaccharide from *Archachatina marginata* exoskeleton through microbial transformation using *S. cerevisiae*.

2. Materials and methods

2.1. Exoskeleton processing of *A. marginata*

Archachatina marginata (*A. marginata*) (snail) procured from the Oluode market, Osogbo metropolis, Osun State, Nigeria, were processed according to the procedure of Felici *et al*., (2020). The snail meats were removed from the shells (exoskeletons), the shells were washed adequately to remove the slime and residual dirt; allowed to air dry and further oven-dried at 40ºC for 6 h. Finally, the dried shells were milled into a powdery form, and sieved using a 5.0 µm mesh size to obtain a homogenous smooth powder.

2.2. Proximate analysis of *A. marginata* exoskeleton

The crude protein, crude fiber, crude lipid moisture, ash and total nitrogen contents of the *A. marginata* shell powder were analyzed using the standard methods of AOAC. (2019).

2.3. Chemical extraction of chitin and chitosan from *A. marginata* shell

Archachatina marginata shell powder was kept in a polythene bag at 28 ± 2ºC for 24 h to allow partial

autolysis; thus facilitating chemical extraction of the chitin and chitosan through demineralization, de-proteinization and deacetylation, according to the methods adopted by Padma Sree *et al*., (2022).

2.3.1 Demineralization of *A. marginata* exoskeleton

About 5 ml of varying concentrations (i.e. 2 %, 3 % and 4 %) of 2 M hydrochloric acid (HCl) was added individually to *A. marginata* shell powder (100 g each), and kept for 16 h at 28± 2ºC. After incubation, the residue was washed and soaked in 25 ml of dist. water for 8 h at 28± 2ºC to neutralize the acidity.

2.3.2 De-proteinization of the *A. marginata* residue

The demineralized samples were transferred into cold 4 % NaOH with a ratio of 5:25 (w/ v) for 20 h at 28± 2ºC. The resulting de-proteinized residue was washed thrice with dist. water and then soaked in 25 ml sterile dist. water for 10 h, to neutralize the alkalinity and obtain pure chitin. The purified chitin was air-dried at 30 ± 2ºC for 24 h until it became crispy, and the resulting chitin flakes were ground into small particles using ceramic mortar and pestle to facilitate the de-acetylation.

2.3.3 Deacetylation of *A. marginata* chitin

Varying concentrations of NaOH (20 %, 40 % and 60 %) with a solid to solvent ratio of 2:20 (w/v) were added for 20 h to remove the acetyl groups from chitin. The residue obtained from de-acetylation of chitin was washed 3 times with 25 ml of sterile dist. water for 30 min., to neutralize the NaOH and improve the chitosan quality. After that, the obtained chitosan was dried at 65± 5ºC for 4 h.

2.4. Colloidal chitin agar preparation

Colloidal chitin was prepared according to the method of Subramanian *et al*., (2020), with a slight modification. The analytical chitin flakes that were processed into powdery form were added slowly to 10N HCl, and then incubated overnight at 4°C with vigorous shaking. The mixture was dispensed into 25 ml of cold 50 % ethanol with rapid stirring at 25°C for 24 h. After incubation, the obtained mixture was centrifuged at 10000 rpm for 20 min. The residue was rinsed thrice with 10 ml of sterile dist. water to neutralize the colloidal chitin (pH 7.0); freeze-dried to obtain colloidal chitin powder, and was kept at 4° C for further analysis. The colloidal chitin agar medium was prepared by incorporating membrane filtered colloidal chitin (0.1 %) into sterile yeast extract agar (2.3 % w/v) after cooling to 45 °C.

2.5. Yeast strains cultivation

Chitin-biotransforming potentials of 2 characterized *S. cerevisiae* strains (KR13 and JH13), which were previously isolated from fermented beverage (palm wine) during the study of Jimoh *et al*., (2012); were screened for chitin degradation by individual inoculation into the center of colloidal chitin agar (CCA) plates, using the point inoculation technique (Sasi *et al*., 2020). After incubation at 37ºC for 24 h, the growing culture media were flooded with phenol red (0.1 %); and the resulting zones of clearance (cm) indicating chitin utilization were measured using a calibrated ruler. The yeast strain that expressed the highest chitin utilization potential was selected for chitooligosaccharide production.

2.6. Chitooligosaccharide biosynthesis

About 3 ml of *S. cerevisiae* KR13 strain (1.1×10^2 cfu\ ml) that expressed the highest chitin utilization capacity was inoculated into a sterile biotransformation broth medium in triplicates, composed of the substrate (5 % *A. marginata* chitin) and mineral salt medium in g\ l [K_2HPO_4 (0.7), KH_2PO_4 (0.3), $MgSO_4.5H_20$ (0.5), $FeSO_4.7H_20$ (0.01), $ZnSO_4$ (0.001), $MnCl_2$ (0.001)]. The media were incubated at 30℃ for 5 d, while analytical chitin served as the control (Subramanian *et al*., 2020). The enzymatic activity and chitooligosaccharide concentration were analyzed at 24 h intervals from the supernatant (crude extract) obtained through centrifugation at 10,000 rpm for 15 min.

2.7. Enzymatic assays

2.7.1. Detection of chitin deacetylase activity (CDA)

The CDA activity was assayed according to the modified procedure of De-hui *et al*., (2011), using a reaction mixture consisting of 1 ml of 1% (w/v) colloidal chitin and 1 ml of the *S. cerevisiae* crude extract, and incubated at 46ºC for 6 h. After incubation, the reacting mixture was centrifuged at 10,000 rpm for 5 min. The resulting supernatant was analyzed for the released acetate using Agilent 6890 GC (Agilent Technologies, California, United States) equipped with a flame ionization detector (FID). An HP-Innowax capillary column (30 m× 0.25 mm× 0.25 mm) equipped with a carrier nitrogen gas at a flow rate of 0.67 ml/ min.; where the oven, injector and detector temperatures were held at 180 ºC, 200 ºC and 220 ºC, respectively. The CDA (1U) was determined as the quantity of enzyme required to release acetate (1.0 mol\ min.) under the GC condition.

2.7.2. Evaluation of the chitinase and chitosanase potentials

The potentials of the chitinase and chitosanase enzymes were evaluated through quantitative analysis of the reducing sugars released from colloidal chitin or chitosan, which were used as substrates for this enzyme assay (Zhu *et al*., 2007). The reaction mixture consisted of 0.5 ml of 1% (w/v) *S. cerevisae* crude extract, 0.5 ml of 1 % colloidal chitin or 0.5 ml of 1 % colloidal chitosan in 1 ml buffer solution that was composed of; 100 mmol/ l citric acid, 200 mmol/ l sodium phosphate, at pH 3-8. The reaction mixture was incubated in a shaking water bath (SW 22, JULABO, Germany) for 30 min. at 36 ºC for the chitinase, and at 56 ºC for the chitosanase. The reaction was terminated by incubation in a water bath at 100 ℃ for 10 min. The quantity of the released reducing sugars in the supernatant was analyzed using Di-nitrosalicylic acid (DNS) reagent; where the absorbance was measured at 540 nm with Microfield UV-Spectrophotometer (MF-752N, England), according to Jimoh and Ajibise, (2017). Accordingly, one unit (1 U) of chitinase or chitosanase activity was defined as the quantity of enzyme that liberated 1 µmol of the reducing sugar as glucosamine (GlcN) per min., using the amino sugar (GlcNAc) standard curve under the same assay conditions, in reference to Subramanian *et al*., (2020).

2.7.3. Chitooligosaccharide deacetylase (COD) potency

Chitooligosaccharide deacetylase (COD) potency was analyzed through a colorimetric method using 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH hydochloride) (Sigma Chemical Co.) reagent, to assay for the amino groups present in the partially deacetylated oligosaccharides. The incubation mixture consisted of 50 µl reaction volume containing [2 mM N, N-diacetyl-chitobiose, 20 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)] at pH 7, and 200 ng of *S. cerevisae* crude extract, and was incubated at 37ºC for 15 min. The metabolic reaction was terminated by boiling the mixture at 100℃ for 4 min., while the product was assayed using MBTH method of Van Wychen *et al*., (2017). Product formation was proportional to the quantity of chitin oligosaccharide deacetylase (COD) enzyme released in the incubation mixture.

2.7.4. Enzymes molecular weights determination

Molecular weights (M) of the chitinase enzymes and their products were analyzed using Size exclusion chromatography (SEC), which is known as Gel permeation chromatography (GPC), in reference to the method adopted by Patkar and Panzade, (2016). The molecular weight (M) of the enzyme was correlated to intrinsic velocity [η] using the Mark-Houwink-Sakurada equation of Pawcenis *et al*., (2016):

$$[\eta] = KMa$$

Where; both values of K and the exponent constant (a) depend on the polymer-solvent system and the temperature.

Using the GPC technique, the average molecular weight values for the polymers and the molecular weight distribution (MWD) were obtained. The characteristic solution parameters including the weight-average of the molecular weights were

calculated and determined. Moreover, additional metrics that clearly describe the solution performance such as weight-average intrinsic velocity [w; average weight radius of gyration (Rgw) and Mark-Houwink constants (a and log K) were determined using a viscometer detector in GPC analysis. Thus, the enzymes exhibited multiple peaks across the molecular range.

2.8. Quantification of the chitooligosaccharide

The synthesized chitooligosaccharide concentration was determined using GC, according to the procedure of Jimoh *et al*., (2021), with a slight modification. The GC conditions used during the analysis included GC software (HP 6890 Powered with HP Chem. Station Rev. A 09.01 [1206]); column type (HP- 5, capillary), column dimensions (30 m × 0.25 µm × 0.25 µm), injection temperature (250°C), detector signal (selected ion mode), oven program (initial temperature at 60°C for 5 min.; first rate at 15°C/ min. for 14 min. that maintained for 3 min.; second ramping at 12°C/ min. for 14 min., which was maintained for 4 min.), and mobile phase (nitrogen) with nitrogen column pressure (30 psi).

2.9. Statistical analysis

The results obtained were analyzed with GraphPad Prism 8.0.1 using one-way analysis of variance (ANOVA) for the proximate composition of *A. marginata* exoskeleton, chitin yield and chitosan yield. The chitinolytic enzymes activities and chitooligosaccharide yield were analyzed using two-way analysis of variance (ANOVA), to determine the significant difference among the means of the triplicate analysis.

3. Results and Discussion

3.1. Proximate composition of *A. marginata* shell

Quantitative analysis of *A. marginata* shell powder was carried out to determine the main chemical components present in the sample. Results obtained in this work revealed a significant difference ($p< 0.05$) in the shell chemical composition (Table 1). Digestible carbohydrate (Nitrogen-free extract) was the main component of the snail shell recording 44.03 %, compared to the other proximate composition. This is attributed to the availability of organic constituents (i.e. polysaccharides and glycoproteins); in accordance with the high carbohydrate content reported by the previous study of Ademolu *et al*., (2015). The high crude fiber content observed in this study was due to the increase in microbiological metabolism of the lipopolysaccharide or utilization of the sugar during the metabolic activities, thus leading to the rise in fiber content (Ogidi *et al*., 2020). The low moisture content (0.06 ± 0.010) and low crude protein content (0.18 ± 0.010) of *A. marginata* shell prevented the microbial contamination and chemical degradation, thus maintaining the stability of the natural product, since high moisture content tends to promote the chemical and metabolic reactions (Ooi *et al*., 2012). These results agreed with the previous studies conducted by Ademolu *et al*., (2015); Nkansah *et al*., (2021), which reported the lack of moisture content (0.00 %) and low moisture content (0.27 ± 0.04 %) in the *A. marginata* shell, respectively. The ash contents of the snail shell represented the quantity and amount of the inorganic components, and the carbon compounds that existed in the form of oxides and salts (Nkansah *et al*., 2021). In this study, the ash content (0.46± 0.01 %) obtained from *A. marginata* shell differed from results of Ademolu *et al*., (2015); Nkansah *et al*., (2021), which reported ash contents of 8.56 % and 96.31 ± 0.01 %, respectively. The relatively low ash content implies that the snail shell contained low quantity of mineral elements, but despite this low content; snail shell ash played an important role in removing heavy metals from the aqueous media, because of its absorbent nature (Nkansah *et al*., 2021). The one-way ANOVA revealed a significant difference among the means of the proximate composition of *A. marginata* exoskeleton.

3.2. Chitin-biotransformation potential of *S. cerevisiae*

S. cerevisiae KR13 strain expressed the widest zone of clearance (2.6 cm) on the colloidal chitin agar indicating its high chitinolytic activity, compared to the other *S. cerevisiae* JH13 strain (2.2 cm). Accordingly, *S. cerevisiae* KR13 was selected for chitin, chitosan and chitooligosaccharide production using the submerged fermentation technique.

Table 1: Proximate composition of *A. marginata* exoskeleton

Parameters	Mean (%)
Moisture content	0.060 ± 0.01
Ash content	0.455 ± 0.02
Crude lipid	14.620 ± 0.00
Crude fiber	40.420 ± 0.38
Crude protein	0.180 ± 0.01
Nitrogen-free extracts (Digestible carbohydrate)	44.200 ± 1.70

Where; the recorded values were significantly different at $p< 0.05$. Data are mean ± SD (standard deviation), n= 3. The statistical level of significance analyzed by a one-way ANOVA is followed by Tukey post hoc pairwise multiple comparisons test

3.3. Chemical synthesis of *A. marginata* chitin and chitosan

During the chemical demineralization of *A. marginata* shell powder, the weight of the demineralized residue obtained from the substrate decreased as the HCl concentration increased, and vice versa (Table 2). This result was observed although the main inorganic constituents ($CaCO_3$ and $CaCl_2$) of the *A. marginata* exoskeletons were removed through demineralization during chemical synthesis, which is attributed to the high efficiency of HCl in removing the minerals. However, a lower concentration of this acid (2 %) produced a higher yield of the demineralized residue. This eventually increased the chitin yield when subjected to de-proteinization using 4 % NaOH solution, in order to remove the proteins from the demineralized residue thus enhancing chitin extraction (Table 2). This result is in agreement with the previous report of Kumar *et al*., (2020), which recorded that the processing temperature, time and concentration of the acid were vital factors that affected the rate of conversion. Further increase in the concentration of NaOH utilized during de-acetylation of chitin decreased the chitosan yield, thus a lower concentration of NaOH (20 %) produced a higher yield of chitosan (Table 3). De-acetylation altered the physical properties of the chitin polymer, to enhance its solubility, flexibility and conferred a positive charge at a neutral pH (Mouyna *et al*., 2020). The one-way ANOVA revealed a significant difference between the chitin and chitosan obtained from the *A. marginata* chitin and analytical chitin medium.

3.4. Chitinolytic enzymes produced during yeast biotransformation

Chitinolytic enzymes such as chitinase; chitosanase, chitin deacetylase and chitooligosaccharide deacetylase biosynthesized by *S. cerevisiae* KR13 strain were identified, and quantified based on their molecular weights and enzymatic activities (Table 4).

Table 2: Demineralization and de-proteinization of *A. marginata* shell

Initial weight of *A. marginata* shell (g)	HCl concentration (%) during demineralization	Weight of demineralized snail shell (g)	NaOH concentration (%) during de-proteinization	Weight of pure chitin (g)
100	2	75	4	48.76 ± 0.176
100	3	70	4	25.00 ± 0.886[a]
100	4	65	4	22.50 ± 1.153[a]

Where; the values followed by the same superscript letters are significantly different at $p< 0.05$. Data are presented as mean ± SD (standard deviation); n=3. Statistical level of significance analyzed by a one-way ANOVA is followed by Tukey post hoc pairwise multiple comparisons test: [a]$p< 0.05$ compared to 2 % HCl and 4 % NaOH, [b]$p< 0.05$ compared to 3 % HCl and 4 % NaOH, [c]$p< 0.05$ compared to 4 % HCl and 4% NaOH

Table 3: Deacetylation of *A. marginata* chitin

Initial weight of pure chitin (g) for deacetylation	NaOH concentration (%) during de-acetylation	Weight of chitosan (g)
20	20	11.23 ± 0.62
20	40	8.33 ± 0.44[a]
20	60	3.67 ± 0.20[a,b]

Where; the values followed by the same superscript letters are significantly different at $p< 0.05$. Data are presented as mean ± SD (standard deviation); n=3. Statistical level of significance analyzed by a one- way ANOVA is followed by Tukey post hoc pairwise multiple comparisons test: [a]$p< 0.05$ compared to 20 % NaOH, [b]$p< 0.05$ compared to 40 % NaOH, [c]$p< 0.05$ compared to 60% NaOH

Table 4: Enzymatic activities and molecular weights of the chitinolytic enzymes

Enzymes	Enzyme activity (Unit/ ml)		Molecular weight (kDa)
	A. marginata chitin medium	Analytical chitin medium	
Chitin deacetylase	0.36 ± 0.029[a]	0.14 ± 0.012[a]	28.2
Chitinase	2.30 ± 0.101[b]	0.69 ± 0.020[b]	120.0
Chitosanase	0.75 ± 0.026[c]	0.27 ± 0.015[c]	52.0
Chitooligosaccharide deacetylase	0.08 ± 0.015	0.14 ± 0.606	45.0

Where; values followed by same superscript letters are significantly different at $p< 0.05$. Data are presented as mean ± SD (standard deviation); n=3. Statistical level of significance analyzed by a two-way ANOVA followed by Tukey post hoc pairwise multiple comparisons test: [a]$p< 0.05$ compared to chitin deacetylase of the analytical chitin medium, [b]$p< 0.05$ compared to chitinase, [c]$p< 0.05$ compared to chitosanase, [d]$p< 0.05$ compared to chitooligosaccharide deacetylase

3.4.1. Activity of chitin deacetylase enzyme

Chitin deacetylase enzyme synthesis was induced by the availability of chitosan and chitin; thus chitin deacetylation interfered with hydrogen bonding, and then transformed the crystal structure of chitin either chemically or biologically, in accordance with Arnold *et al*., (2020). The availability of chitin deacetylase enzyme during the biotransformation indicated the expression of chitin deacetylase genes by *S. cerevisiae* KR13. These genes were used for the biosynthesis of intracellular chitin deacetylases within the periplasm, in addition to the production of extracellular chitin deacetylases into the culture medium (Grifoll-Romero *et al*., 2018). The extracellular enzyme hydrolyzed the acetyl groups of the substrates (i.e. chitosan or chitin) using multiple attack mechanisms (Kaczmarek *et al*., 2019); where the enzyme bounded to the chitin chain caused several sequential deacetylations before binding to another chain (Aragunde *et al*., 2018). As a result, such de-acetylation presumably modified the physicochemical properties of the chitin polymers, and regulated their binding to the associated macromolecules (Latańska *et al*., 2021). This might affect the properties and architecture of the chitin-based macromolecular assemblies (Behr and Ganesan, 2022). There was a significant difference between the chitin deacetylase enzyme obtained from the *A. marginata* chitin and the analytical chitin medium using a two-way ANOVA.

3.4.2. Potentials of the chitinase and chitosanase enzymes

The activity of *S. cerevisiae* KR13 strain to depolymerize *A. marginata* chitin and chitosan was demonstrated by the availability of chitinase and chitosanase enzymes in the biotransformation crude extract. Although these enzymes catalyzed the hydrolysis of the glycosidic bonds; however, their substrate specificity differed with the chitin and chitosan bonds that were being hydrolyzed (Aragunde *et al*., 2018). The availability of the enzyme subsites that interacted with N-acetylglucosamine (GlcNAc) residues in the substrate from the non-reducing to the reducing ends allowed the chitin deacetylases to identify a sequence of the GlcNAc units in the substrate (Martínez-Cruz *et al*., 2021). Where, a single GlcNAc unit undergone deacetylation, thus the resulting chitosan possessed a regular deacetylation pattern more than the chitosan treated with hot NaOH (Arnold *et al*., 2020). Accordingly, chitooligosaccharides were produced when the chitin was depolymerized or hydrolyzed by chitinases, while de-N-acetylation of chitooligosaccharides and chitin yielded chitosan (Kaczmarek *et al*., 2019). The two way ANOVA revealed a significant difference between chitinase and chitosanase enzymes' obtained from *A. marginata* chitin and the analytical chitin medium.

3.4.3. Potency of chitooligosaccharide deacetylase

The availability of chitooligosaccharide deacetylase in the biotransformation crude extract showed the degrading potential of *S. cerevisiae* KR13 strain, which led to enzymatic modification of the chitooligosaccharides to generate products with desired chain arrangements, in agreement with results of the previous study conducted by Kaczmarek *et al*., (2019). Using a two way ANOVA, there was no significant difference between chitooligosaccharide de-acetylase enzyme obtained from *A. marginata* chitin and the analytical chitin medium.

3.5. Yield of chitooligosaccharides

Chitooligosaccharides derivatives including chitohexose; chitopentose, chitotetrose, chitotriose, chitobiose and chitosan with varying concentrations were identified and quantified in the biotransformation medium (Table 5). Varying concentrations of the recovered chitooligosaccharides were attributed to the chitinases mechanisms of degradation. Chitinases have the capacity to remain associated with the substrate for a new cleavage to occur, as the polymer substrate slides through the substrate-binding cleft (Sørlie *et al*., 2020).

Table 5: Yields of the chitooligosaccharides

Analytical chitin Medium		*A. marginata* chitin medium		
Retention time (min.)	Amount (mg/ ml)	Retention time	Amount (mg/ ml)	Chitooligosaccharide
9.485	0.191	9.669	0.714	Chitohexose
10.862	0.166	11.494	0.571	Chitopentose
12.209	0.032	12.511	0.762	Chitotetrose
13.682	0.020	13.715	0.629	Chitotriose
15.009	0.017	15.921	0.286	Chitobiose
20.721	0.002	20.221	0.301	Chitosan
Chitooligosaccharide yield	0.402 ± 0.028		3.385 ± 0.096	

Where; total values are significantly different at $p< 0.05$. Data are presented as mean ± SD (standard deviation); n=3. Statistical level of significance analyzed by a two-way ANOVA is followed by Tukey post hoc pairwise multiple comparisons test

This implies that the enzymes have different binding interactions for the different sequences on the substrate. These reactions showed multiphasic kinetic model that changed the product profiles obtained during hydrolysis, and at the end of each of these phases the product mixtures differed considerably (Liaqat and Eltem, 2018). Furthermore, production potential of the chitooligosaccharides by *S. cerevisiae* KR13 strain was revealed by its ability to synthesize chitin deacetylases required for bioconversion of chitin to chitosan; through enzymatic hydrolysis of the N-acetyl linkage thus converting GlcNAc to GlcN (Schmitz *et al*., 2019). *S. cerevisiae* KR13 strain synthesized chitosan derivatives that effectively enhanced the biosynthesis of chitooligosaccharides, compared to the chemically synthesized chitosan. Accordingly, these reactions overcome the solubility limitations of chitosan production at high acid and alkali concentrations, which mean that chitooligosaccharides production at neutral pH will be favorable. Using a two-way ANOVA, there was a significant difference between chitooligosaccharide yield obtained from *A. marginata* chitin and the analytical chitin medium.

Conclusion

The availability of chitin deacetylase enzyme in the culture medium indicated the biotransformation potential of *S. cerevisiae* KR13 strain, which catalyzed the enzymatic deacetylation of chitin to chitosan. Thus, the production of a higher yield of chitosan with higher molecular weight authenticates the superiority of the enzymatic reactions over the chemical processes. Furthermore, the expression of chitinase and chitosanase enzymes required for catalytic hydrolysis of the glycosidic linkages within the chitin and chitosan led to the synthesis of chitooligosaccharides, which confirms that *S. cerevisiae* KR13 strain had the potential to synthesize chitinolytic enzymes with different substrate specificities. Activity of the chitooligosaccharide deacetylases also confirmed the ability of *S. cerevisiae* to further enzymatically modify the chitooligosaccharides to yield products with desired chain arrangements. The chitooligosaccharides possessed the properties required to be safely used within the pharmaceutical and biomedicine applications. However, further future research can optimize the preparation methods required to produce low molecular weights enzymes and to facilitate their use in the biomedical applications.

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Conflict of interest

The authors declare no conflict of interests.

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Ethical approval

Ethical approval was not required.

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