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Short Communication

USE OF 3,5-DINITROSALICYLIC ACID REACTION TO STUDY THE CHITOSAN HYDROLYSIS

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Abstract

Chitin, one of the most abundant polymer in the biosphere, and its main derivative, chitosan have many applications in which the hydrolysis of the polymer chain has to be performed. To control the hydrolysis reaction a method to assess the course of reaction is required. Among other methods, DNS (3,5-dinitrosalicylic acid) assay is most used. The main drawback of the classical DNS method is that the heating step, i.e. boiling in water, required glass test tubes. The miniaturization of the method, i.e. performing the reaction in ELISA plates have the technical problem of heating the microtitter plates. This work present an improved DNS method, in which the DNS reaction is performed in ELISA plates, heated on thermoblock. The new improved method was applied to the hydrolysis of chitosan with chitinase.

Keywords: DNS, reducing sugars, glucosamine, N-acetil-glucozamine, microtiter plate assay

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1. Introduction

It is well known that the modern society has growing needs for materials, energy, chemicals to make advanced final products to improve the quality of life. On the other side, there is a growing concern regarding the global warming, consumption of the fossil resources and disposal of wastes. In this context, more attention was addressed to the renewable raw materials as a source for creation of the advanced final products. Today, many waste products are not used properly used to obtain valuable chemicals. In this category enter also chitin. This material is a polymer synthetized in huge quantities, especially by marine organisms. It is estimated that chitin is the second most abundant polymer synthetized on biosphere, with an annual production of 10^{10} – 10^{12} tons, after cellulose[1]. The annual worldwide commercial production of crustaceans exceed 10 million tons[2]. The huge amount of waste products resulted from processing of seafood become a major environmental issue, as about 45% of the mass of marine organisms used as seafood (shrimps) is waste. About one third of this garbage is chitin[3]. As chitin and its deacetylated derivative chitosan are a renewable resources, these materials become the source of numerous applications in agriculture, foods, waste water treatment, textiles, cosmetics, pharmaceuticals, medicine, biotechnology and many other fields[4,5].

For many applications, after the extraction of the polysaccharide from the waste materials, the polymeric chain has to be hydrolyzed to smaller fragments and in some cases until monomers. Classical hydrolysis is performed with acids, but in order to make the hydrolysis process more environmentally friendly the hydrolysis reaction is realized with hydrolytic enzymes - carbohydrases (glycoside hydrolases, O-glycosidases) – that belong to the class of hydrolases EC 3.2.1.-[6]. Both, hydrolysis with acids or with enzymes, has to be monitored. Most of the assays for determination of hydrolysis of carbohydrate polymers are based on analysis of reducing sugars. Among these methods, the Somogyi-Nelson method[7,8], based on copper and arsenomolybdate reagents and the DNS method[9], are the most used. Less frequently used are the method with potassium ferricyanide[10], sodium 2,2'-bicinchoninate[11] or p-hydroxybenzoic acid hydrazide[12]. Most of the above mentioned methods, as are based on chemical reaction of reducing sugars with some chemicals, require a heating step. This is why in the majority of the published articles the experiments are performed in glass tubes that can be warmed in boiling water. In some previous papers [13,14] we have described the attempt to perform the DNS reaction in ELISA microtitter plates, by performing the heating step in a microwave oven. In this study we have adapted the DNS reaction to microtitter plates, realizing the heating step in a thermoblock. The method was applied to study the hydrolysis of chitin and chitosan.

2. Materials and Methods

2.1. Chemicals and Instruments

The chemicals used in this study were acquired from Sigma Aldrich or Carl Roth: glucosamine hydrochloride (Sigma Aldrich, # G1514), N-acetil-glucosamine (CarlRoth, # 8993.2), glucose anhydrous (Scharlau, #GL01250500), maltose (Carl Roth, #8951.1), galactose (Carl Roth,#4987.2), lactose (Carl Roth, #8921.1), sodium hydroxide (Sigma Aldrich, # 367176), 3,5-dinitrosalicylic acid (DNS; Sigma Aldrich, # D0550) phenol (Carl Roth,# 0040.1), potassium sulphite (Carl Roth, # 7995.1), potassium tartrate (Sigma Aldrich, # 243531), potassium sodium tartrate tetrahydrate (Carl Roth, #8087.1), potassium hydroxide (Carl Roth , # P747.2), ELISA plates were acquired from Sarstedt (#82.1581),

Optical density of the reaction product was determined by using of a Tecan Sunrise microplate reader (Tecan Trading AG, Männedorf, Switzerland) with a Magellan Data Analysis software, A HLC Heating-Thermo Mixer MHR 11 (DITABIS - Digital Biomedical Imaging Systems AG, Pforzheim, Germany) was used for heating of the samples to 100°C .

2.2. Stock solutions

To perform the DNS reaction, the following variants were prepared: ***Recipe 1:*** 1% DNS, 30% potassium sodium tartrate tetrahydrate, 4N sodium hydroxide; ***Recipe 2***: 1% DNS, 1% sodium hydroxide, 0.2% phenol, 0.05% potassium sulphite; *DNS solution **3** **Recipe 3***: 1% DNS, 30% potassium tartrate, 0.5% sodium hydroxide, 0.5% potassium hydroxide, 0.2% phenol.

The following stock solutions were used: 10 mM glucose solution; 10 mM galactose solution; 10 mM lactose solution; 10 mM maltose solution; 50 mM glucosamine hydrochloride solution, 50 mM N-acetil glucosamine solution. All stock solution were prepared in distillated water.

2.3. Methods

2.3.1. DNS reaction in microtitter plates

The reaction of DNS reagent with the solutions containing reducing sugars were performed in microtitter plates. The total volume of DNS reagent (one of the three recipes) was (usually) 100 µL and the maximum volume of the containing the analyte was also 100 µL. The heating step was realized on a microplate heat block. The plates were covered with a plastic cover, to reduce as much as possible the evaporation process.

2.3.2. Calibration curve for reducing sugars

To realize the calibration curves, various volumes of stock solutions (glucose, maltose, galactose, glucosamine, N-acetyl-glucosamine) ranging from 1 to 100 µL were mixed with 100 µL DNS reagents (one of the three Recipes). ELISA plates were incubated for 30 minutes, at 100°C in thermomixer. To avoid evaporation of solutions the plates were covered with a foil. After cooling the plates at room temperature, optical density was measured at 540 nm in the plate reader. All experiments were performed at least in duplicate.

3. Results and Discussions

The mains goals of this study were to miniaturize the DNS method for reducing sugars, i.e. to perform this method in microtitter plates and to optimize this method for the evaluation of hydrolysis of chitin and chitosan.

Although the DNS methods was miniaturized to ELISA plates[13,14], the heating step was performed in the microwave oven, that made the assay to be unpleasant for technical point of view. In this study the heating phase was realized in an ELISA thermic block.

Three types of recipes were tested, in order to make the DNS reagent as simple as possible, but also as sensitive and accurate as possible. In Figure 1 there is presented a comparison of the optical densities of the same solution of glucosamine (GN) when different recipes of DNS reagents were used.

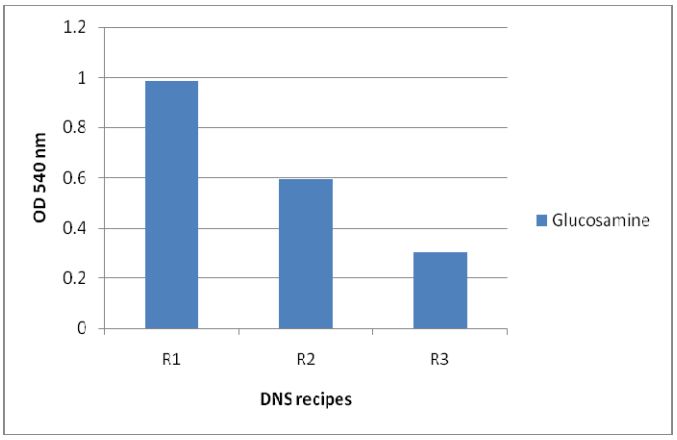

Figure 1 The influence of DNS solution compounds on reaction products absorbance. The concentration of glucosamine and N - acetil - glucosamine was 6.25 mM

Based on the results presented in Figure 1, in the rest of the experiments the DNS reagent presented in recipe 1 was used.

Several mono- and di-saccharides (glucose, galactose, lactose, maltose, glucosamine, N-acetil glucosamine) were tested and standard calibration course were realized (Figure 2 presents some of the standard curves).

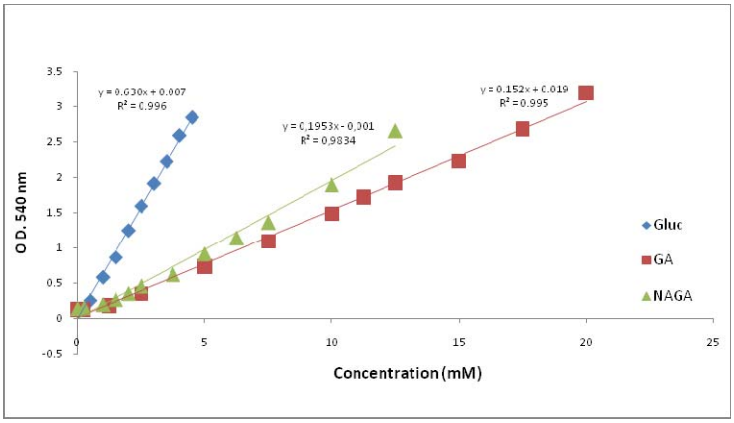

Figure 2 Calibration curves for glucose, glucosamine (GA) and N-acetil-glucosamine (NAGA)

The conclusion that can be drown from Figure 2 and from **Table 1** is that the DNS reagent has different sensibility when the reducing sugars are in their native state, i.e. the –OH groups are not functionalized or blocked, comparing with the case when these –OH groups are substituted with amino or N-acetyl-amino groups.

Table 1 Values of slope, interception and regression coefficient for reducing sugars. Limits of detection (LOD) and quantification (LOQ) are expressed in mM reducing sugars.

Sugar	**a**	**b**	**R^2**	**LOD**	**LOQ**
Glucose	0.6309	0.0073	0.9967	0.317498	0.962115
Galactose	0.6384	0.0048	0.997	0.303391	0.919367
Lactose	0.6589	0.0203	0.9929	0.468246	0.418928
Maltose	0.638	0.006	0.9961	0.346015	1.048531
Glucosamine	0.1953	0.001	0.9834	1.558106	4.721534
N-acetil-glucosamine	0.152	0.0197	0.9958	1.799821	5.494004

The slopes of the standard curves on neat mono- or di-saccharides are higher than when the monosaccharides units contain an amino or N-acetyl-amino group. This information has to be taken into account when the DNS assay hast to be applied to assess the hydrolysis of various types of polysaccharides. If during the hydrolysis of the polysaccharide will result fragments with the reducing ends belonging to neat carbohydrate, like glucose, galactose, manose, then the standard curve can de realized with glucose. If the fragments resulted from

hydrolysis of the polymeric chain will produce reducing sugars that have amino or N-acetly-amino groups, then the standard curves has to be realized with the specified monomers.

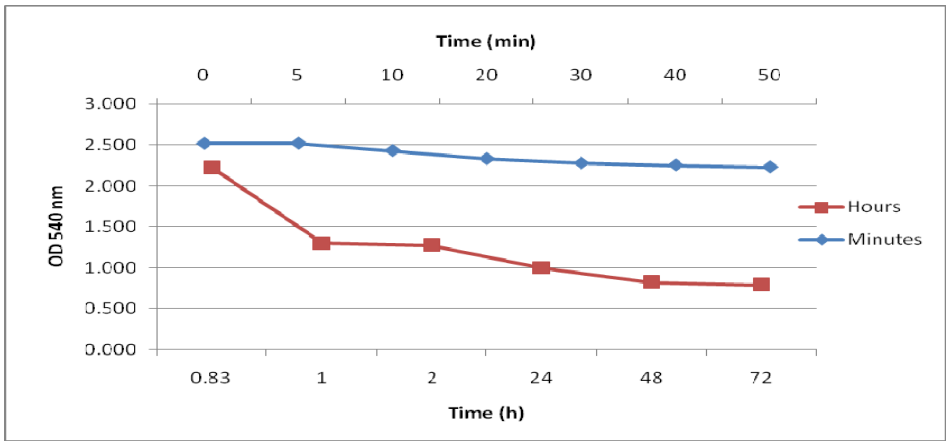

Figure 3 The stability of the colour of the reaction product. Lower horizontal axis, corresponding to the red experimental points, is in hours, while the upper horizontal axis, for the blue experimental points, is in minutes.

As one of the objective of this work was to set-up a method useful for the study hydrolysis of chitosan, the rest of experiments were realized with glucosamine (GN). In Figure 3 there is presented the stability of the colored reaction product. The color intensity of the final reaction product do no decrease in one hour with more than 3%, but after 72 hours, the color fade with more than 60%. It is recommended to read the plates as soon as 30 minute after the heating step of the reaction was ended.

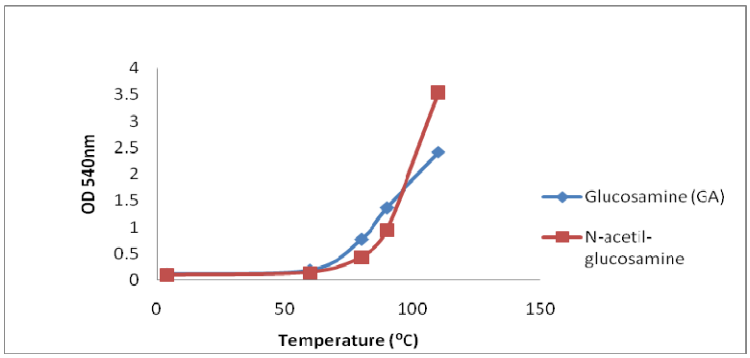

Figure 4 Influence of temperature on reaction product absorbance

From the variation of the color intensity of the reaction product as a function of the temperature of the reaction, we may assume that it is important to perform the heating of the plates at 100°C, as the results presented in Figure 4 show. As these are preliminary results of

a wider study of optimization of DNS method, there are no results at temperature higher than 100°C.

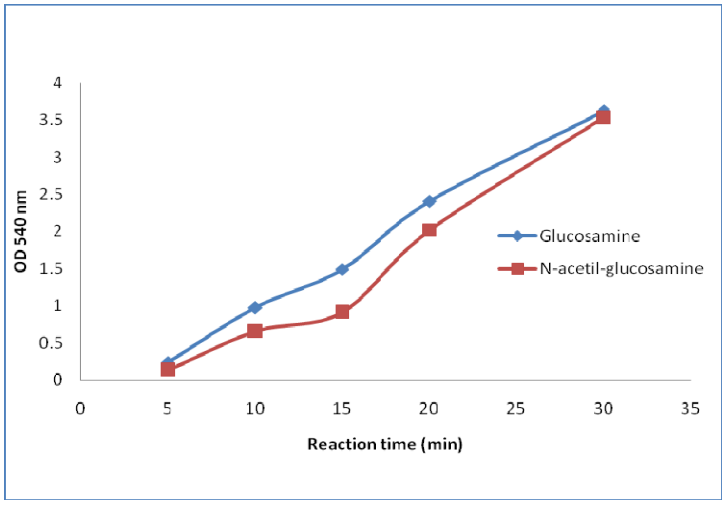

Figure 5. The influence of the time of reaction (DNS reagent with glucosamine and B-acetyl-glucosamine, respectively) upon the color intensity of the final product.

Although based on the preliminary results presented in Figure 5, it become obvious that after 30 minutes of the reaction between DNS reagent and the reducing sugar solution, a plateau was not reached, due to practical reasons, a 30 minutes reaction time (at 100°C) was considered for the rest of experiments.

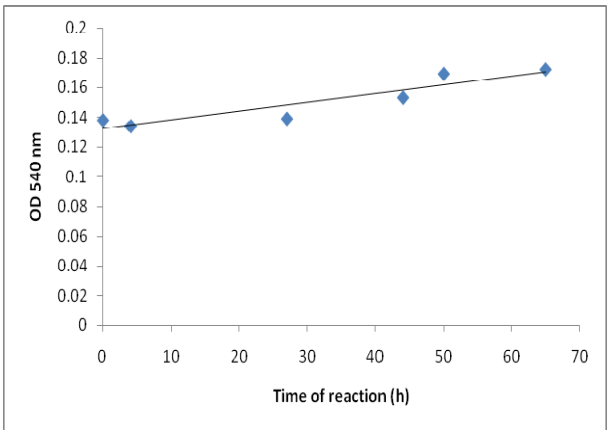

Figure 6 DNS assay for reaction between chitinaze and chitosan

As an application of the DNS method improved to be realized on ELISA microplates, in Figure 6 there is presented the hydrolysis of a chitosan sample with chitinase.

Although the DNS method here presented, in comparison with the previous methods also performed in ELISA plates[13,14] requires a much longer time to be realized, have the advantage to be simpler. In the previous methods, the heating step was performed in vessel with water, in a microwave oven and for technical point of view this was an unpleasant operation. The present method, although longer, is technically simpler. We also expect that the method with the thermic block instead of microwave oven to be more accurate and even sensitive, after an optimization process based on design of experiments approach.

4. Conclusions

The classical DNS method was miniaturized to be performed in ELISA plates. The heating step was performed on ELISA thermic block. The main inconvenient of this new procedure is the rather long time of the reaction (30 min) that has to be realized, in comparison with a previous method when the heating step was realized on microwave oven. The new proposed method was applied to the hydrolysis of chitosan with chitinase.

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