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Preparation and Characterisation of Chitosan from *Penicillium chrysogenum* Thom for Decolourisation of Congo Red Dye in Aqueous Solution

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ABSTRACT: This work investigated the removal efficiency of Congo red dye (CRD) from aqueous solution using chitosan prepared from the biomass of *Penicillium chrysogenum* Thom. CRD is a benzidine - based anionic diazo dye known to be carcinogenic at low concentration. Chitosan was prepared from the mycelium of *P. chrysogenum* through the process of deproteination and deacetylation; and the chitosan was characterised using Fourier transform infrared spectroscopy (FT-IR) analysis through which the degree of deacetylation (DD) was estimated. Adsorption study was carried out in a slurry batch system at ambient temperature (28 ± 1°C). The percentage yield of chitosan from the fungus was 12.2% while 82.4% DD was achieved. The FT-IR spectrum showed that chitosan contained amide carbonyl band as a measure of *N*-acetyl group, alkyl groups and hydroxyl groups; the spectrum of *P. chrysogenum*-based chitosan corresponded to the pattern of the commercial chitosan used as control. The commercial and *P. chrysogenum*-based chitosan attained decolourisation efficiency of 95.2% and 84.6% respectively, which gives the indication that chitosan from *P. chrysogenum* biomass, can effectively remove CRD from aqueous solution.

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Chitin is a natural polymer. In addition to cellulose and starch, it is one of the three most abundant polysaccharides in nature. It ranks second to cellulose as the most plentiful organic compound on earth. Chitin is the main component of the cell walls of fungi such as *Penicillium* species (Wang *et al*., 2007), *Mucor absidia* and Mushrooms (Ahila *et al.*, 2014; Latha and Suresh, 2013). It is also a major component of the exoskeletons of arthropods, such as the crustaceans (e.g. crab, prawn and shrimp), the insects (e.g. ants, beetles, cockroach and butterflies) (Rinaudo 2006; Wanule *et al.,* 2014), and the beaks of cephalopods (e.g. squids and octopus). Chitin has proven useful for several medical and industrial purposes. Chitin is composed of β (1-4) linked 2 – acetamido -2- deoxy – β – D – glucose (N – acetylglucosamine). It is often considered as cellulose derivative, even though it does not occur in organisms producing cellulose (Dutta *et al.,* 2004). Chitin may be described as cellulose with one hydroxyl group on each monomer replaced by an acetyl-amine group. This allows for increased hydrogen bonding between adjacent polymers, giving the polymer increased strength. Chitosan, a more water-soluble source of chitin is gotten by the treatment of chitin with 40-50% sodium hydroxide, this process is called deacetylation. The major source of commercial chitosan is the crustaceans where chitosan extraction is gotten by a major process of deproteination, demineralisation, bleaching with hydrogen peroxide or hypochlorate and deacetylation. However, crustaceans are limited to seasons. Several alternative sources of chitin have been suggested, including insects, antarctic krill and diatoms but no significant progress has been made in these areas (Kannan *et al.,* 2010). An alternative source of chitin/chitosan are fungi as they contain chitin and some have chitosan in their cell wall. Recent advances in fermentation technology have suggested that large-scale culturing of an organism that contains chitin/chitosan might be an attractive route to the production of this polymer. Reports showed that this organism can be readily cultured on simple nutrients (Miyoshi *et al*., 1992). It was also reported that fungal mycelia have lower levels of inorganic materials compared to crustacean shells, and no demineralisation treatment is required during processing (*Wu et al*., 2005).

Chitosan finds application in so many areas including water and wastewater treatment. Wastewater is any water produced by different domestic and industrial activities. It contains various inorganic, organic and biological contaminants that are detrimental to the environment. These contaminants can create health hazards if discharged without proper care and treatment into water bodies. One of the major

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contaminants of industrial wastewater is dyes (colorants) such as Congo red (sodium salt of benzidine diazo bis-1- napthtylamine -4- sulphonic acid). It has been known to cause allergic reactions, and can be metabolised to benzidine, a human carcinogen (Rouf *et al*., 2015). The reactive cleavage of azo linkage is known to be responsible for the formation of toxic amines in wastewater (Joshi *et al.,* 2004). Congo red dye (CRD) is also detrimental to plants on exposure (Sawhney and Kumar, 2010) and it is therefore important for wastewater containing dyes such as Congo red to be treated before disposal. Decolourisation of dye in aqueous solution by chitosan occurs via adsorption process; adsorption has been shown to have an edge over other treatment methods due to its sludge free operation, and thorough removal of dyes even from dilute solutions (Negrulescu *et al.,* 2014). Many organisms like *Klebsiella, Bacillus, E. coli, Pseudomonas, Enterobacter*, fungi and yeasts have been studied for the decolorisation of Congo red (Chen *et al.,* 2003; Jalandoni-Buan *et al.,* 2010). Other non-conventional, low price adsorbents such as banana and orange peels (Annadurai *et al.,* 2002), rice husks (Ong *et al.,* 2009) and activated carbon (Hema and Arivoli, 2007) have also been used for the removal of azo dyes from aqueous solutions; but not all of these adsorbents can effectively remove anionic dyes due to their hydrophobic or anionic surfaces (Negrulescu *et al*., 2014). Therefore, this study investigates the use of chitosan prepared from *P. chrysogenum* as more efficient adsorbent for the decolourisation of CRD in aqueous solution.

MATERIALS AND METHODS

Collection and preparation of Chitosan Samples: The cultures of *P. chrysogenum* were obtained from fungal bank of the Department of Plant Biology and Biotechnology, University of Benin, Benin city, Nigeria. The cultures were expanded in potato dextrose broth and incubated for three days at 28^{o}C; the mixture was then transferred to potato dextrose agar and allowed to stay for five days at ambient temperature (28±1^{o}C). Three plugs of the fungal culture were mixed with 200ml yeast peptone dextrose medium containing 0.2% yeast extract, 1% peptone and 2% glucose in a 500ml Erlenmeyer flask, and the flask was incubated at 28^{o}C without shaking. After the period of incubation, the mixture was filtered and washed with distilled water until a clear filtrate was obtained. The fungal biomass were dried in an oven at 60^{o}C and ground to powder using mortar and pestle. The resultant powder was thereafter stored in a desiccator at room temperature for the extraction of chitin (Wu *et al*., 2005).

The dried *P. chrysogenum* powder was suspended in 1M sodium hydroxide solution in the ratio of 1:30 (w/v), and the mixture was autoclaved at 121^{o}C for 15 minutes to remove the protein content of the biomass (deproteination). Then the mixture was centrifuged and the alkaline insoluble fraction was collected and washed with distilled water. The residue was boiled with 2% hydrochloric acid for 6 hours at 95^{o}C. It was then centrifuged, and the supernatant was adjusted to pH of 9 using 2M sodium hydroxide to precipitate the chitosan in the supernatant. The resulting solution was centrifuged and the residue (chitosan) was washed with distilled water and dried at 60^{o}C to constant weight (Ebrahimzadeh *et al*., 2013; Wu *et al.,* 2005).

Characterisation of Chitosan Powdered Sample: The application of chitosan is determined by the quality parameters obtained by characterisation. These include solubility, viscosity, degree of deacetylation etc. The yield of the prepared chitosan was determined by gravimetric method, the solubility test was carried out by dissolving 1% of chitosan in 1M hydrochloric acid. The viscosity of the soluble chitosan in hydrochloric acid was determined at 29^{o}C using Brookfield digital Viscometer (model LVDV-1) as follow: about 20ml of the solution was placed in a sample container; a standard spindle attached to the viscometer was immersed into the sample and rotated at 260rev/min. The viscosity was read on the equipment-displayed screen. Fourier transform infrared (FT-IR) analysis was carried out using Shimadzu FT-IR (8300BX model) spectroscope.

Calculation of Degree of Acetylation (DA) and Degree of Deacetylation (DD): The degree of deacetylation (DD) was calculated using FT-IR spectrum, and the calculation procedures are based on absorbance ratios of various spectral bands using the following equations:

$$Degree\ of\ acetylation\ (DA) = \left[\frac{\left({}^{A_{CO}}/_{A_{OH}}\right)\times 100}{1.33}\right] ..1$$

$$Degree\ of\ deacetylation\ (DD) = 100 - DA\ ..2$$

Where: A_{CO} is absorbance of amide carbonyl band as a measure of *N*-acetyl group content; A_{OH} is absorbance of hydrogen band as an internal standard because hydroxyl groups are stable group in chitin and chitosan structures which are not affected by hydrolysis of chitin to chitosan. 1.33 is the ratio of A_{CO}/A_{OH} ($CO\ at$ 1657cm^{-1} and $OH\ at$ 3468cm^{-1}) for fully *N*-acetylated compound (Ebrahimzadeh *et al*., 2013).

FT-IR measurements in most cases have been performed in the transmission mode with sample contained in potassium bromide tablets, and the spectrum is based on the vibrations of the atoms of a molecule. The percentage transmittance (%T) values are converted to absorbance (A) through the following expression:

$$A = 2 - \log(\%T) \ldots 3$$

Where, A =Absorbance of the solution and %T = %Transmittance

An IR spectrometer usually records the energy of the electromagnetic radiation that is transmitted through a sample as a function of the wavenumber or frequency. The energy of each peak in an absorption spectrum corresponds to the frequency of the vibration of a molecule. The total spectrum is analysed by an interference process and converted into the frequency or wavenumber range by means of a mathematical process known as the Fourier transform (Kumirska *et al*., 2010).

In a batch adsorption system, 200ml of CRD solution (40mg/L) was mixed with 100mg of *P. chrysogenum*-based chitosan powder in Erlenmeyer flask. A control flask containing 100mg of a commercial chitosan and 200ml of 40mg/L CRD was set up alongside. The mixture in the flasks was agitated at room temperature (28±1^{o}C) in an orbital shaker (model THZ-82) at a speed of 160rpm for three hours. Samples were collected at predetermined time interval and filtered. The absorbance of the filtrate was measured in a T-80 model UV/VIS spectrophotometer at a wavelength of 530 nm. The residual dye concentrations were interpolated from a standard calibration curve relating the absorbance to the concentration of CRD previously obtained from preliminary study. The equilibrium time was determined from the resulting data. The percentage decolourisation efficiency was calculated using the following equation:

$$\%\ decolourisation\ = \frac{C_o - C_t}{C_o} \times 100 \ldots\ldots 4$$

Where C_o = Initial dye concentration (mg/L), C_t = Dye concentration at time t (mg/L).

RESULTS AND DISCUSSION

The yield of chitosan extracted from *P. chrysogenum* was 12.2%. This is slightly lower than the 20-30% yields obtain from other species of *Penicillium* such as *P. waksmanii*, *P. viridicatum*, *P. aurantiogriseum* and *P. citrinum* (Ebrahimzadeh *et al*., 2013), but higher than 5.7% obtained from *P. chrysogenum* in the study of Wang *et al*. (2007). 12.49% yield of chitosan from *Mucor rouxii* was also reported by Wu *et al*. (2005). The variation in percentage yield of chitosan as reported in literature depends on the source and type of culture medium used for the preparation of the chitosan (Wang *et al.,* 2007; Pochanavanich and Suntornsuk, 2002). The solubility of chitosan is an important parameter for its quality because higher solubility indicates better chitosan (Patria, 2013). The solubility of chitosan obtained in this study was 76% for *P. chrysogenum* -base chitosan and 89% for commercial chitosan. It was ascertained that solubility is directly proportional to DD due to hydrogen ions on the amine group which make chitosan to easily interact with water through hydrogen bonding (Patria, 2013). The viscosity of *P. chrysogenum*-based and commercial chitosan are presented in Table 1.

Table 1: Quality parameters for *P. chrysogenum*-based chitosan

Parameters	*P. chrysogenum* -based chitosan	Standard chitosan
Yield (%)	12.2	-
Solubilty (%)	76	89
Viscosity cP	3.7	3.2
Degree of deacetylation (%)	82.4	83.3
Decolourisation Efficiency (%)	84.6	95.2

It has been reported that viscosity depends on temperature and that as the viscosity increases, the acetyl content in the chitosan solution increases. This is due to solubility of acetyl which turns the solution to gel-like especially at high temperature (Patria, 2013). The FT-IR spectrum of chitosan prepared from *P. chrysogenum* was observed to be similar to that of the commercial chitosan. The spectra were obtained within a frequency (λ) range of 400 – 4000 cm^{-1}. The spectra had characteristic bands that show the presence of functional groups such as hydroxyl group (OH stretch at 3200- 3600cm^{-1}), amide carbonyl functional group of acetyl component (C=O stretch at 1640-1680cm^{-1}), esters (C-O stretch 1150-1300cm^{-1}), and alkyl groups (C-H stretch at 2850-3000), (C-H bend indicated at1415-1465cm^{-1}). The characteristic bands in the spectrum of *P. chrysogenum*-based chitosan are in conformity with the pattern exhibited by the standard chitosan. The FTIR spectra for the standard and *P. chrysogenum*-based chitosan are

presented in Figure 1. Another parameter used to characterise chitosan is degree of deacetylation (DD), and this can be done using various methods such as nuclear magnetic resonance (NMR) spectroscopy, potentiometric titration methods, UV-VIS spectroscopy and infrared spectroscopy methods. The most precise measurements of DD in chitosan require sophisticated equipment such as NMR spectrometer but the equipment is not available at many laboratories.

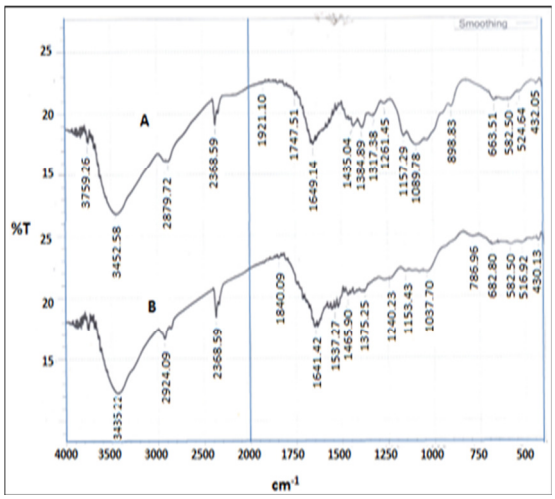

Fig 1: FTIR spectra (A) standard chitosan (B) chitosan from *P. chrysogenum*

Infrared (IR) spectroscopy is another widely used analytical techniques available for characterisation of chitin and chitosan (Kasaai, 2010). The calculation of DD is obtained from the baseline drawn across the absorbance peaks of the amide carbonyl band and the hydroxyl group as an internal standard. From the FTIR spectra in Figure 1, *P. chrysogenum*-based chitosan had a DD of 82.4% while the commercial chitosan had 83.3%, which is slightly lower than the stipulated ≥85% DD inscribed on the label of the container. Degree of deacetylation as an important quality parameter that is mostly used in the charaterisation of chitosan. This shows the percentage of acetyl groups that can be removed from chitin during the preparation of chitosan. High DD indicates that the acetyl group in chitosan is low. A low acetyl group brings a stronger interaction between the ions and hydrogen bonds of chitosan (Patria, 2013).

The percentage decolourisation efficiency of CRD (Figure 2) shows 95.2% and 84.6% removal by the commercial and *P. chrysogenum*-based chitosan, respectively. The commercial chitosan had a rapid adsorption of the dye within the first 40 minutes, and the process steadily progressed to attain equilibrium at 120 minutes, while the *P. chrysogenum*-based chitosan had a slower adsorption of the dye until equilibrium was reached at 140 minutes. The slight deviation in the pattern of adsorption could be as a result of the purity and DD of the adsorbents.

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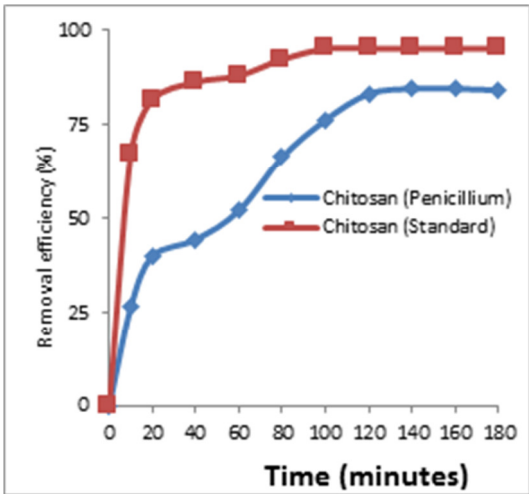

Fig 2: Percentage of Congo red dye adsorbed onto Chitosan

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