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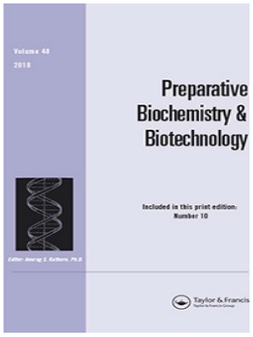

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Kinetic and thermodynamic investigations of cell-wall degrading enzymes produced by *Aureobasidium pullulans* via induction with orange peels: application in lycopene extraction

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ABSTRACT

The production of cell-wall degrading enzymes (*CWDE*) such as cellulase and pectinase by *Aureobasidium pullulans* NAC8 through induction using orange peels was investigated for the potential application of these enzymes in the extraction of lycopene from tomato skin, waste, and paste (*SWP*). The *CWDE* was then immobilized via entrapment in alginate beads for lycopene extraction and the kinetic/thermodynamic properties of the free and immobilized *CWDE* investigated. The optimum production of *CWDE* occurred at pH, temperature, and orange peel concentration of 6.0, 50 °C, and 2.0% (w/v), respectively. The values obtained for some kinetic and thermodynamic parameters such as E_d^*, $t_{1/2}$, ΔG_d^*, and ΔH_d^* indicate that both free and immobilized cellulase and pectinase were thermostable between 40 and 50 °C. Maximum lycopene extracted from the tomato *SWP* was 80 ± 2.4 mg/kg, 42 ± 1.3 mg/kg and 60 ± 1.2 mg/kg, respectively, using the immobilized *CWDE*. The entrapped *CWDE* was able to extract lycopene with yields of 58 ± 4.2, 51 ± 1.2 and 57 ± 4.2% for tomato *SWP* respectively after the fifth cycle. Using orange peels for the induction of *CWDE* by *A. pullulans* offers a unique and cheaper approach to obtaining thermostable multi-enzyme complexes employable for easy lycopene extraction from tomato *SWP*.

KEYWORDS

Cellulase; pectinase; lycopene; submerged fermentation; *Aureobasidium pullulans* NAC8; orange peel; tomatoes

Introduction

Lycopene, an acyclic tetraterpenic hydrocarbon, is a natural antioxidant found mostly in tomatoes and it is known to confer the reddish color on them. The potentials of lycopene to act as potent antioxidants stems from the fact that they contain 11 conjugated double bonds.[1] The consumption of fresh tomatoes is known to have great health benefits such as lowering the risks associated with certain cancer types.[2] Lycopene have attracted a lot of interest in the food and pharmaceutical industry where they are used as natural colorants as well as in food fortification mainly because of their non-toxicity, biodegradability, and strong color.[3–5] The lycopene present in peels is almost five times higher than the pulp, thus making the peels a much sought-after source for the extraction of lycopene.[6] The Industrial extraction of lycopenes from tomatoes involves the use of solvents which has deleterious effects downstream such as the requirements for a large amounts of solvents. This leads to occupational, health, and environmental hazards.[7] Also, there are challenges with the low yields when solvents only are used for lycopene extraction, this is due to the inability of solvent molecules to access the rigid tomato peel tissues. The lycopene is deeply enmeshed in the chromoplast membrane structure and the inability of the solvents to enter this structure and hence solubilize it makes solvent extraction undesirable.[8] To bypass these challenges, the use of enzymes (mostly cellulase and pectinase) that can degrade the cellulose and pectin present in the tomato peels have been exploited.[6,9,10] These enzymes hydrolyze the main polysaccharide components of the plant structures where the pigment is stored, thus aiding in the release of intracellular contents. Other approaches used for the extraction of lycopene include the use of super critical-CO_2 and ultrasonication.[10–12] The advantages offered by enzyme-assisted extraction of lycopene over solvent-based approach is that it (i) is not time-consuming, (ii) occurs at ambient temperature, and (iii) provides higher yields.[13] Lycopene extraction using mixed enzyme preparations containing both cellulase and pectinase has been reported and oftentimes, these enzymes are obtained commercially and then applied to the tomato peels or paste in a sequential manner.[10] In this study, cell-wall degrading enzymes (*CWDE*) were produced by submerged fermentation using orange peels as a substrate. Ismail[14] reported that orange peels are rich in cellulose and pectin and can be used as a cheap substrate for the

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Table 1. Definition of enzyme units.

Enzyme	Definition
Cellulase	One unit of *cellulase* activity is defined as the amount of enzyme releasing 1.0 µmol glucose equivalent from carboxymethyl cellulose per minute under the standard assay condition
Pectinase	One unit of *pectinase* activity is defined as the amount of enzyme releasing 1.0 µmol galactoronic acid equivalent from pectin per minute at room temperature under the standard assay condition
Xylanase	One unit of *xylanase* activity is defined as the amount of enzyme releasing 1.0 µmol xylose equivalent from the substrate per minute at room temperature under the standard assay condition
Amylase	One unit of *alpha amylase* activity is defined as the amount of enzyme releasing 1.0 µmol maltose equivalent from the starch per minute at room temperature under the standard assay condition

induction of multienzyme complexes by *Aspergillus niger*. In that same study, the author reported that some cell-wall degrading enzymes such as cellulase, pectinase, xylanase, and alpha-amylases were produced after induction of *A. niger* with the orange peel wastes. In this study, the cell wall degrading enzymes produced by induction of *Aureobasidium pullulans* with orange peels under optimum conditions was immobilized by entrapment in alginate. Enzyme immobilization via entrapment in alginate is a cheap and easily adaptable technique compared to other known techniques like cross-linking and adsorption.[15–17] Recently, *A. niger* pectinase was immobilized on alginate and its kinetic and thermodynamic properties investigated[18] while immobilized pectinase was used for the extraction of lycopene from waste tomato peels.[19] Ladole et al.[16] used cross-linked cellulase and pectinase in lycopene recovery from tomato peels but in this report, alginate was employed for enzyme immobilization for lycopene extraction from tomatoes. To the best of our knowledge, this is the first report where cellulase and pectinase were co-immobilized on alginate for the extraction and recovery of lycopene from tomato peels. In this study, the entrapped *CWDE* was further used for the extraction of lycopene from tomato skin, waste, and paste over several reaction cycles. The use of immobilized enzymes for lycopene extraction offers a better advantage than free enzymes because of its improved enzymatic activities, better stability, and reusability.[15,20–22] This is the first study of its kind where carbohydrases for lycopene extraction were produced by *A. pullulans* by induction with orange peels. *Aureobasidium pullulans* is a yeast-like fungus that is very ubiquitous as it is found in almost every habitat such as decayed plant litters,[23] on various crops and fruits, etc.[24,25] This fungus is renowned for producing very important industrial extracellular enzymes such as laccase, fructosyltransferase, cellulase, amylase, etc.[23,26,27] The fungus had been applied in biodegradation of textile dyes[22] and other remarkable products like antibiotics.[28] This fungus was selected for the production of these cell-wall degrading enzymes (*CWDE*) by harnessing its potential to utilize orange peels wastes (rich in pectin and cellulose) as cheap substrates.

Enzymes for industrial applications must display a higher degree of thermal resistance and in this study, the thermodynamic and kinetic characteristics of the *CWDE* was also investigated to provide information on its application industrially especially in the food industry. It is noteworthy to mention that no report of the investigation of the thermodynamic and kinetic characteristics for *A. pullulans* cellulase and pectinase exists in literature; hence, this is the first study of its kind.

Materials and methods

Materials

Bovine serum albumin, Coomassie blue G-250, carboxymethylcellulose (CMC), beechwood xylan, polygalacturonic acid, butylated hydroxytoluene, corn starch, and dinitrosalicylic acid were obtained from either from Sigma Chemical Company, St. Louis, USA or Merck Company, Darmstadt, Germany. All solvents used in this study were of analytical grade with purity greater than 95%. Apparently healthy and ripened *L. esculentum* and Tomato pastes (Gino™ Brand) were collected from the local market in Ile-Ife, Osun State, Nigeria. Orange peel waste was obtained from local orange sellers in the Obafemi Awolowo University, Ile-Ife, Nigeria. They were washed with distilled water, dried, milled, sieved with a mesh size of 10 µm and stored at 4 °C prior to further use. The chemical composition of the orange peels such as cellulose, hemicellulose, and pectin content was determined according to methods described by Liu et al.[29]

Methods

Microorganism and culture conditions

The fungus used in this study, *A. pullulans* NAC8, was isolated from soil containing decayed plant litters. The gene sequences were deposited in the GenBank database with an accession number KX020331.[23] The fungus was stored on Malt Extract agar (MEA) in a McCartney bottle at 4 °C and sub-cultured every 72 h.

Submerged fermentation for cell-wall degrading enzyme (CWDE) production

CWDE production was carried out by submerged fermentation in a 100 ml flask containing 20 ml of the medium. The media composition was a modified version described by Ismail:[14] $NaNO_3$ – 2.0 g/L, $MgSO_4.7H_2O$ – 0.5 g/L, KCl – 0.5 g/L, K_2HPO_4 – 1.0 g/L, and $FeSO_4.7H_2O$ – 0.01 g/L. The orange peel concentration was initially varied between 0.1% and 10% w/v. The sterilized medium was inoculated with 5

mm agar plug cut from actively growing regions of the fungi on MEA. The inoculated medium grew without agitation. The samples were withdrawn every 24 h, centrifuged at 4000×*g* and the supernatant served as the *CWDE* source. Cellulase, pectinase, xylanase, and alpha-amylase assays were carried out as well as protein concentration.

Cell-wall degrading enzyme assays

The assay for the *CWDE* was carried out using the 3, 5 dinitrosalicyclic acid method (DNSA).[30] The enzymatic reaction mixtures for cellulase, pectinase, xylanase, and α-amylase activities contained 500 µl of 1% (w/v) of carboxymethyl cellulose, pectin, beechwood xylan, and starch respectively in 0.5 mM acetate buffer pH 5.5 and 200 µl of the enzyme. The reaction mixture was incubated at 25 °C for 20 min and reaction terminated using 3, 5 dinitrosalicyclic acid reagent. The reaction mixture was then boiled for 10 min and the reducing sugars present quantified appropriately. A control was set up such that the enzyme was pre-incubated with the DNSA before adding the substrate.[31–34] All experiments were conducted in triplicate measurements. Table 1 shows the definition of the enzyme units.

Determination of protein concentration

Protein concentration was determined using the method described by Bradford[35] with bovine serum albumin as the standard protein.

One factor at a time optimization for production of cell wall degrading enzymes

Optimization of *CWDE* (cellulase and pectinase) production by *A. pullulans* was by one-factor-at-a-time (OFAT) and the factors investigated for its effects on *CWDE* production were pH (4–9), temperature (20–80 °C), inoculum size (5–30 mm), and days of incubation (1–8). For the effect of pH the medium was adjusted to the desired pH with 1 N HCl or NaOH, respectively. Enzyme assays and protein concentration for each factor determined in this optimization studies were carried out in triplicate measurements.

Immobilization by entrapment in alginate

To entrap the *CWDE* the method of Ragu and Pennnathur[36] was modified and used. Briefly, 0.8 g of the sodium alginate was mixed with 1 ml of the CWDE to reach 8% (w/v) final alginate concentration. This mixture was aspirated into a 5 ml syringe and then extruded gently into a petri-dish containing 50 ml of 5% (w/v) calcium chloride solution. Enzyme-entrapped beads were formed immediately and the reaction mixture was left for one hour. The beads were collected by decantation, rinsed in excess of 0.5 mM acetate buffer, pH 5.5 and stored at 4 °C prior to further use. The beads containing the entrapped enzyme were used in the hydrolysis of carboxymethylcellulose and pectin for several cycles. Typically, 0.1 g of the immobilized CWDE was incubated in 0.5 ml of the substrate for 15 min. The beads were collected via decantation while supernatant obtained was separately collected in a clean test tube. DNSA reagent (0.5 ml) was added to the supernatant and boiled using methods described in section "Cell wall degrading enzyme assays." The beads obtained was rinsed in acetate buffer (pH 5.5, 0.5 mM), drained and reused for another round of catalysis.

The immobilization yield is calculated thus:

$$\text{Immobilzation Yield} = \frac{\text{Activity of Immobilized Enzyme}}{\text{Activity of Free Enzyme}} \times 100$$

Effect of pH and temperature on free and immobilized cellulase and pectinase

The effect of temperature on the free and immobilized cellulase and pectinase activity was determined by incubating the reaction mixture at various temperatures (20–90 °C) for 20 min. The residual activity was assayed under the conditions described above. To study the effect of pH on the free and immobilized cellulase and pectinase, the substrates (carboxymethylcellulose and citrus pectin for cellulase and pectinase, respectively) were prepared in 0.5 mM buffers of different pH values (4–9). The buffers used include acetate (4–5), phosphate (6–7), and Tris-HCl (8–9). The effect of pH on enzyme activity was assayed as described in earlier sections.

Kinetic and thermodynamic studies

Enzymes for industrial applications must display some measure of thermostability. Enzyme thermostability involves both kinetic and thermodynamic properties. Kinetically, the thermal denaturation of enzymes is described by first-order kinetics using the following equation:

$$v_{d=\ k_d\ E}, \tag{1}$$

where v_d is the rate of enzyme denaturation, k_d is the first-order constant, and E is the enzyme concentration.[18] The k_d is directly related to the Arrhenius equation:

$$k_d = Ae^{-E_d/RT}, \tag{2}$$

A is the pre-exponential factor, E_d is the activation energy of denaturation, R is the universal gas constant, and T is the absolute temperature.

The k_d was estimated from the plot of the slopes of the plot of In (μ) against temperature (T). µ is the activity coefficient, and it is best described as the ratio of enzyme, E, to the enzyme concentration at the beginning of being exposed to a defined temperature, E_0, $(\frac{E}{E_0})$.

In this study, k_d was obtained from the slopes of the plot of In (μ) against T. Other kinetic parameters such as half-life $(t_{1/2})$, decimal reduction time (D) values were estimated using the equations described by

$$t_{1/2} = \text{In}\ 2/k_d \tag{3}$$

$$D = \text{In}10/k_d. \tag{4}$$

The enzyme half-life defined as the time after which the enzyme activity was reduced to one-half of the initial activity while the decimal reduction time is defined as the time of enzyme exposition at a known temperature to maintain 10% residual activity.[18,37] Another important thermal denaturation kinetic parameter is the sensitivity factor (z-value), which is defined as the increase in temperature that results in the reduction of the D-value by one logarithmic cycle was estimated in this study from the slopes of log$D$ vs. T (°C).

From Eq. (2), the activation energy of the irreversible enzyme inactivation (E_d) was derived from the slope of the plot of Ink_d *against* $1/T$ for temperatures ranging from 40 to 70 °C. Other important thermodynamic parameters for free and immobilized cellulase and pectinase inactivation were determined using equations described below:

$$\Delta H_d^* = E_d - RT \tag{5}$$

$$\Delta G_d^* = -RT\mathrm{In}\left(\frac{k_d h}{k_b T}\right) \tag{6}$$

$$\Delta S_d^* = \frac{\Delta H^* - \Delta G^*}{T}. \tag{7}$$

The overall entropy of activation and enthalpy of activation for the thermal denaturation were obtained from the below equation:

$$\mathrm{In}\left(\frac{k_d}{T}\right) = \mathrm{In}\left(\frac{k_b}{h}\right) + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{R}\left(\frac{1}{T}\right) \tag{8}$$

ΔH_d^* , ΔG_d^*, and ΔS_d^* are the activation enthalpy, Gibbs free energy, and entropy, respectively. Boltzmann constant (k_B) is 1.38 × 10^{-23} J^{-1} K^{-1}, Plank's constant (h) is 6.63 × 10^{-34} J^{-1} s^{-1}, and the universal gas constant (R) is 8.314 J^{-1} mol^{-1} K^{-1}.[18]

The activation energy, *Ea*, was determined from the slopes of Arrhenius plot from the optimal temperature data (see Arrhenius equation in Eq. (1)), In (k) vs. $1/T$, where k is the reaction rate, T is the temperature in Kelvin, and A is the pre-exponential factor.

$$\mathrm{In}\ (k) = \mathrm{In}\ A - \left(\frac{Ea}{R}\right)^{1}/_{T} \tag{9}$$

Preparation of *Lycopersicon esculentum*

Apparently healthy and ripe tomatoes (maturation: 65 ± 3 days; stored at 4 °C) were rinsed with distilled water and then hand peeled as described by Zuorro and Lavecchia.[38] The tomato skins were peeled off, homogenized, and stored in air-tight containers prior to further use at 4 °C. The pulp without the skin and the seed were separately blended and regarded as tomato wastes. Tomato paste was used as purchased without prior processing.

Extraction of lycopene

The extraction of lycopene was carried out with slight modification in the procedure suggested by Fish et al.[39] *Lycopersicon esculentum* skin, waste, and paste were subjected for solvent extraction of lycopene using tri-solvent mixture consisting of hexane:acetone:ethanol (2:1:1) and 1% butylated hydroxytoluene. The tomato skin, waste, and paste (1 g) was weighed into 50 ml conical flasks that contained 10 ml of 0.5 mM acetate buffer pH 5.0. The mixture was stirred for 5 min then 20 ml of the tri-solvent mixture was added. Incubation was at 25 °C for 2 h under low-speed agitation (100 rpm). The mixture was allowed to stand for 5 min for the separation of phases. From the upper phase, 3 ml was collected and further diluted using the tri-solvent mixture. The absorbance of this was measured using a UV visible spectrophotometer at the 503 nm. The lycopene concentration (mg/kg), in each extract, was calculated using the formula described below.[3] Experiments were carried out in triplicate measurements.

$$\text{Lycopene}\left(\frac{\text{mg}}{\text{kg}}\right) = \frac{31.2 \times \text{Dilution factor} \times A_{503\text{nm}}}{\text{gram of sample}}$$

Enzyme-aided extraction of lycopene

The reaction mixture for the enzyme-aided extraction of lycopene from *the skin, waste, and paste* was prepared using 1 g of the *L. esculentum* waste, skin, and peel and 1 g of alginate-entrapped *CWDE* or 1 ml of the *CWDE* (for the free enzyme) as appropriate. Incubation of the reaction mixture occurred at 50 °C for 2 h. The alginate-entrapped *CWDE* was removed from the reaction mixture, rinsed in 0.5 M acetate buffer pH 5.5, and then used for another cycle of reaction. The residues obtained after removal of the alginate-entrapped *CWDE* were subjected to solvent extraction and incubation for 2 h at 25 °C under low-speed agitation (100 rpm). The lycopene was then quantified using methods described above.[3] To investigate the effect of different concentrations of tomato skin, waste, and paste on the lycopene yield, the tomato skin, waste, and paste concentration were varied between 1 and 5 g and incubated with 1 g of the alginate-entrapped *CWDE*. The process for lycopene extraction and quantification is as discussed in the above section. Lycopene quantification was carried out in triplicate measurements.

The effect of the different amounts (g) of immobilized enzyme on the lycopene extraction was investigated by incubating 1 g of the tomato skin, waste, or paste with various amount of immobilized enzyme (1–5 g) for 1 h. The process for lycopene extraction and quantification was carried out as discussed in the above section. Lycopene assay and quantification was carried out in triplicate measurements

Analysis of lycopene

The lycopene extracted via enzyme-assisted approach and just solvents only were analyzed using Fourier transform infrared spectroscopy (FTIR).[10,15] Briefly, the extracted lycopene was blended with potassium bromide and then

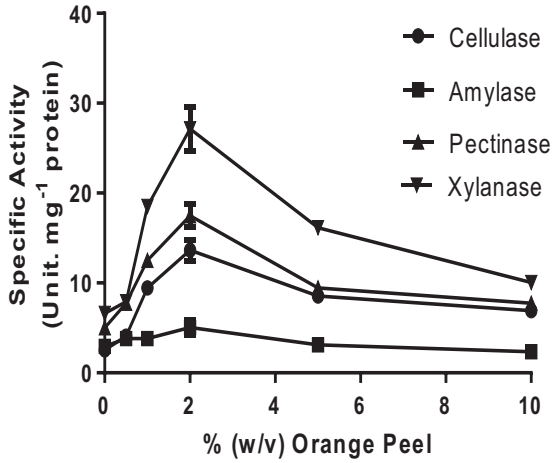

Figure 1. Cell-wall degrading enzymes produced by *Aureobasidium pullulans* NAC8 using orange peels at various concentrations (w/v), and the pH, inoculum size, and temperature of the medium were maintained at 5.0, 5 mm, and 25 °C, respectively. Error bars represent standard deviation ($n = 3$).

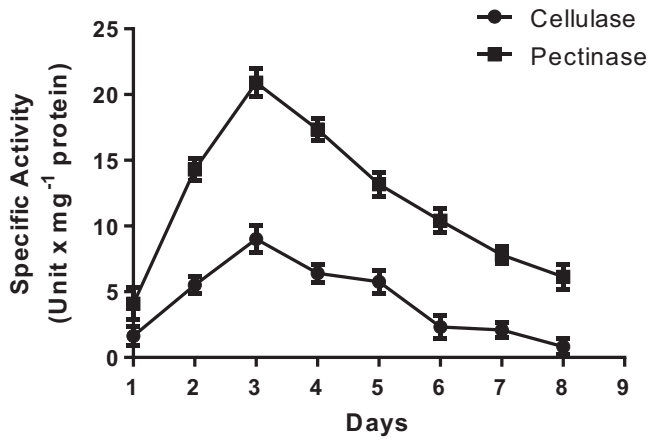

Figure 2. Effect of incubation time (days) on cellulase and pectinase production by *Aureobasidium pullulans NAC8*. Error bars represent standard deviation ($n = 3$). The orange peel concentration was 2% (w/v), and the pH, inoculum size, and temperature of the medium were maintained at 5.0, 5 mm, and 25 °C, respectively.

screwed to form pellets using hydraulic gauges. The spectral data were collected in the range of 4000–400 cm using an FTIR spectrophotometer (Shimadzu FTIR-8400S).

Data analysis

The data obtained in the experimental process were analyzed with the analysis of variance (ANOVA) with a level of significance: $p < 0.05$, using the GraphPad Prism version 6.0 (GraphPad Inc.)

Result and discussion

Induction of cell-wall degrading enzymes and optimization of cellulase and pectinase production

Orange peels are often disposed of as waste leading to adverse environmental and health challenges. The orange peels used in this study were collected as wastes for the production of cell wall degrading enzymes such as cellulase and pectinase. These enzymes were now employed for the breakdown of cellulose and pectin present in tomato tissues for lycopene release. Several authors have used orange peels as a source of producing a cocktail of enzymes mainly because of the orange peels are a cheap and readily available source of cellulose and pectin.[14,29] In this study, the chemical composition of orange peels revealed that cellulose (20%), hemicellulose (22%), and pectin (29%) were present. Li et al.[14] reported that the cellulose, hemicellulose, and pectin concentration of the orange peels used were 14, 21, and 22%, respectively, while Ismail et al.[29] reported that cellulose, hemicellulose, and pectin concentration were 17, 39, and 22%, respectively. The presence of these fibers in the orange peel necessitated its potential use as a cheap and readily available source of substrates for the induction of the *CWDE* by *A. pullulans* NAC8. Orange peels at 2% (w/v) maximally induced the production of *CWDE* by *A. pullulans* as pectinase, xylanase and cellulase were the most expressed enzymes while relatively negligible amylolytic activities were observed (Fig. 1).

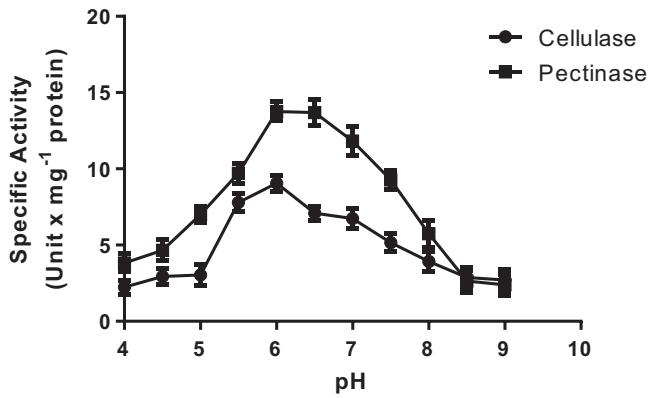

Figure 3. Effect of pH on cellulase and pectinase production by *Aureobasidium pullulans* NAC8. The orange peel concentration was 2% (w/v), and the inoculum size and temperature of the medium were maintained at 5 mm and 25 °C, respectively. Error bars represent standard deviation ($n = 3$).

This finding is supported by the fact that starch is present in diminishing amounts in the orange peel and this same conclusion was reached by Ismail et al.[14] The optimum value of 2% (w/v) of substrate for maximum enzyme production was not in agreement with the report of Ahmed et al.[40] where the optimum value for substrate concentration for pectinase production by *A. niger* was 4%. Increasing the substrate above 2% led to a gradual decline in the enzyme expression by the fungi used in this study. The maximum production of the *CWDE* by *A. pullulans* occurred on the third day of incubation (Fig. 2), pH of 6.0 (Fig. 3), the temperature of 50 °C (Fig. 4), and inoculum size of 10 mm (Fig. 5). The values obtained for some parameters investigated in this study are in agreement with those obtained for pectinase production by *A. niger* and *Penicillum oxalicum* PJ02 induced with orange peels.[29,40] *Aspergillus niger* produced pectinase optimally at 5th day of incubation, incubating temperature and pH of 30 °C and 5.5, respectively, while *P. oxalicum* had optimum temperature of 37 °C. Other authors have reported that *Fusarium oxysporum* and *Aspergillus aculeatus* are more thermostable and have an optimum temperature that varies between 60 and 65 °C.

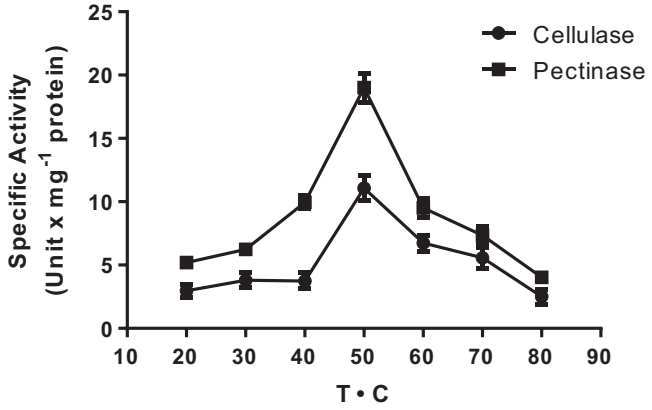

Figure 4. Effect of temperature on cellulase and pectinase production by *Aureobasidium pullulans* NAC8. The orange peel concentration was 2% (w/v), and the inoculum size and pH of the medium were maintained at 5 mm and 5.0, respectively. Error bars represent standard deviation ($n = 3$).

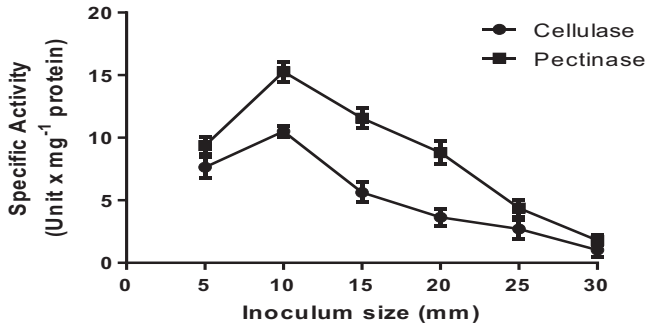

Figure 5. Effect of inoculum size on cellulase and pectinase production by *Aureobasidium pullulans* NAC8. The orange peel concentration was 2% (w/v), and the pH and temperature of the medium were maintained at 5.0 and 25 °C. Error bars represent standard deviation ($n = 3$).

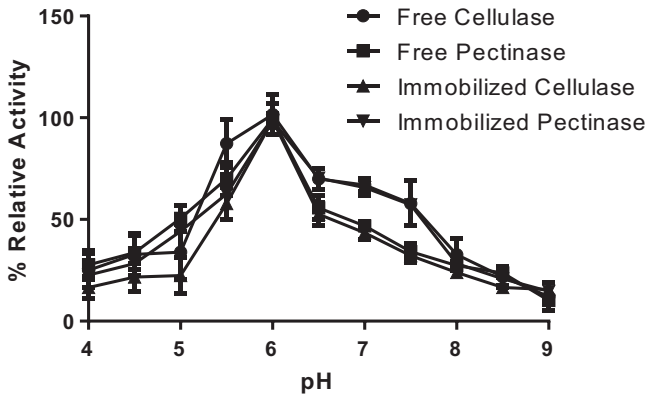

Figure 6. Effect of pH on free and immobilized pectinase and cellulase produced under optimized conditions. Error bars represent standard deviation ($n = 3$).

The optimum temperature and pH for the free and immobilized were 50 °C and 6.0, respectively (Figs. 6 and 7). The optimum temperature obtained in this study was similar to that obtained for *A. aculeatus*.[18]

Kinetic and thermodynamic stabilities of free and immobilized Cell-Wall degrading enzymes

Enzyme thermostability involves both thermodynamic and kinetic stabilities; and, in this study, the thermodynamic and

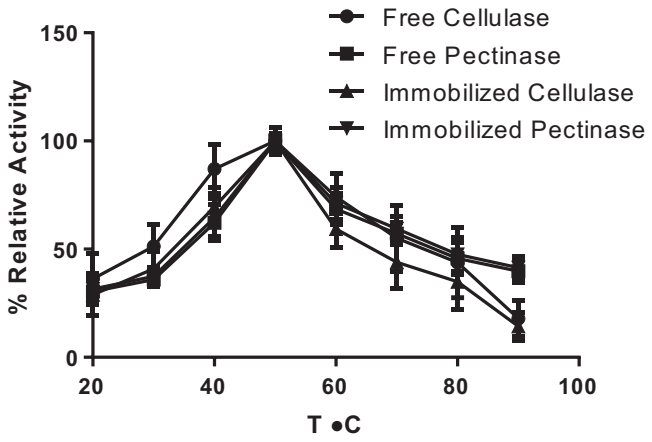

Figure 7. Effect of temperature on free and immobilized pectinase and cellulase produced under optimized conditions. Error bars represent standard deviation ($n = 3$).

kinetic stability studies of immobilized and free cellulase and pectinase were investigated. Enzymes for industrial applications must show resistance to thermal denaturation; hence, this study investigated the potentials of both the free and immobilized forms of cellulase and pectinase to overcome thermal resistance at temperatures ranging from 40 to 70 °C. It was observed from the semi-log plots of $In\ (\mu)$ against T, (where μ is the activity coefficient and T is the temperature in Kelvin), that the activity of the free and immobilized cellulase and pectinase followed a typical first-order enzyme deactivation pattern (Fig. 8a–d). This observation was in agreement with the findings of Pal and Khrum[20] and Olievera et al.[18] for free and immobilized xylanase and pectinase, respectively.

Kinetic parameters for enzyme thermal denaturation

Table 2 illustrates the summary of the major kinetic parameters determined for the free and immobilized cellulase and pectinase for *A. pullulans*. The values obtained for the first-order thermal denaturation constant, (k_d) decreased as the temperature increased while the half-life $(t_{1/2})$, increased as the temperature increased. This observation is not unexpected as it meant that, as the process of thermal denaturation increased, this simultaneously led to a gradual decline in the thermostability. A conclusion easily drawn from k_d and $t_{1/2}$ values obtained in Table 2 for free and immobilized cellulase and pectinase is that thermostability was achieved around 50 °C. The results obtained in this study for *A. pullulans* cellulase and pectinase (free and immobilized) agrees with that of immobilized exoploygalacturonase from *Penicillium notarum*[41] and free and immobilized pectinase from *A. aculeatus*.[18] The cell-wall degrading enzymes produced in this study was used in lycopene extraction from tomato wastes; hence, it is important to express the first-order thermal denaturation reactions in terms of the D and z-values. These values are often required by most food processing industries.[18] At temperatures between 40 and 50 °C, good thermostability was observed as it took more time (i.e., 794–548, 576–343, 1097–767, and 177–124 min) for immobilized cellulase, free cellulase, immobilized pectinase, and

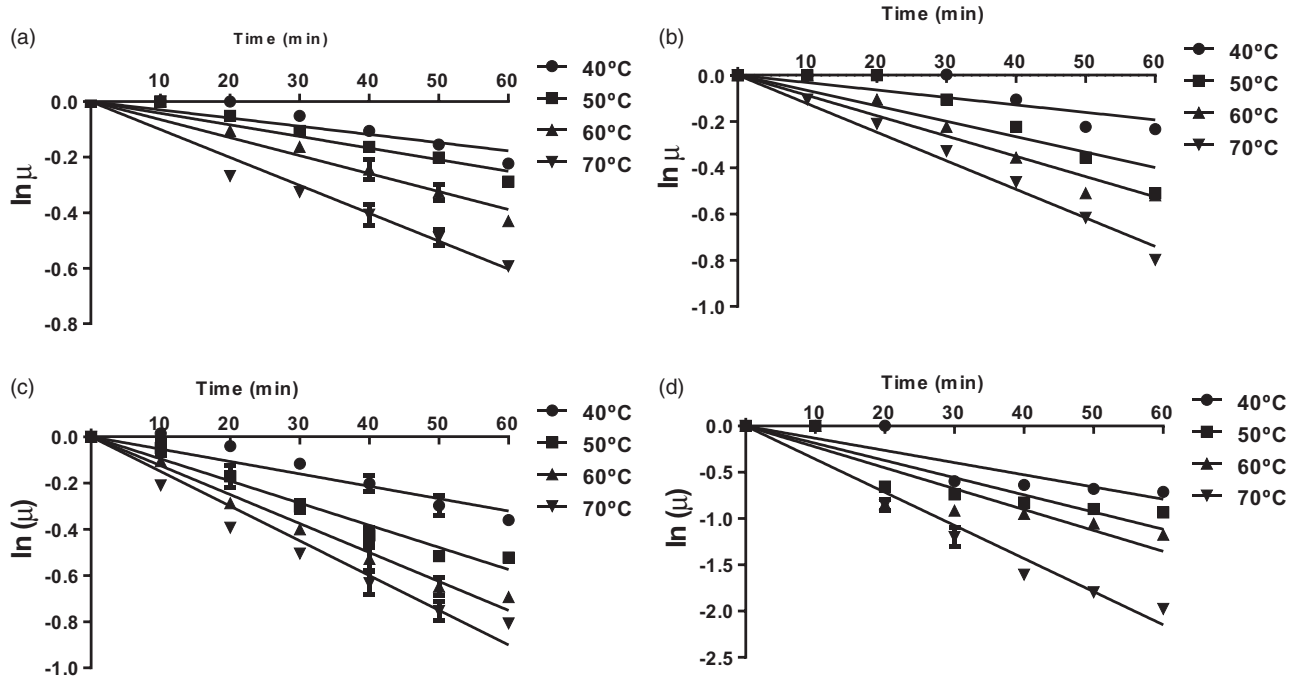

Figure 8. (a) Semi-log plots of thermal denaturation of free cellulase. (b) Semi-log plots of thermal denaturation of immobilized cellulase. (c) Semi-log plots of thermal denaturation of free pectinase. (d) Semi-log plots of thermal denaturation of immobilized pectinase.

Table 2. Kinetic properties of free and immobilized cellulase and pectinase from *Aureobasidium pullulans* NAC8.

T (K)	k_d (min)$^{-1}$	R^2	$t_{1/2}$ (min)	*D*-value (min)	*Z* (°C)
Immobilized cellulase					
313	0.0029	0.97	239	794	54.7
323	0.0042	0.98	165	548	
333	0.0065	0.99	107	354	
343	0.0102	0.97	68	226	
Free cellulase					
313	0.0040	0.97	173	576	61.2
323	0.0067	0.99	104	343	
333	0.0099	0.98	70	233	
343	0.0123	0.98	56	187	
Immobilized pectinase					
313	0.0020	0.95	347	1097	36.5
323	0.0031	0.99	231	767	
333	0.0045	0.97	165	512	
343	0.0150	0.98	46	153	
Free pectinase					
313	0.0130	0.98	53	177	70.9
323	0.0186	0.96	37	124	
333	0.0226	0.95	31	102	
343	0.0358	0.98	19	64	

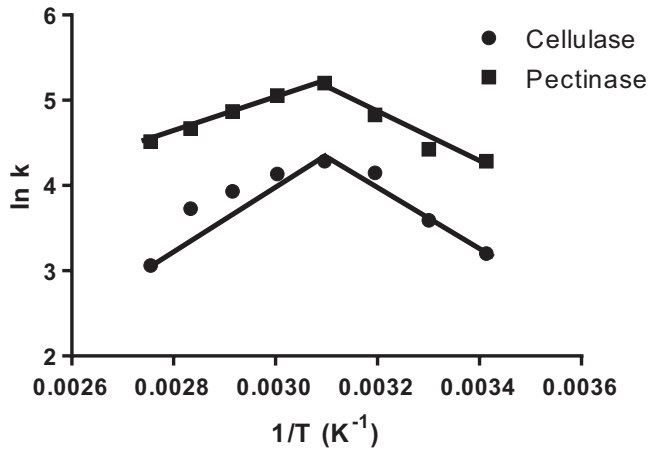

Figure 9. Arrhenius plot (ln *k* vs 1/*T*) to determine activation energy (Ea) for cellulase and pectinase.

free pectinase, respectively) to reach a 10% reduction of the initial activity. As temperatures increased above 50 °C, there was a rapid drop in the *D*-values, this observation was similar to the decline in the *D*-value noted at 50 °C for immobilized and free pectinases.[18,42] The *z*-values obtained for the free and immobilized cellulase means that the decimal reductions of their *D*-values needed temperature increases of 54.7 and 61.2 °C, respectively, while the *z*-values for the for the free and immobilized pectinase indicated that the decimal reductions of their *D*-values required temperature increases of 35.5 and 70.9 °C, respectively. It was observed that immobilization increased the *z*-values (with the exception if immobilized pectinase) as this negates the findings of Oliviera et al.[18] and Abdel Wahab et al.[42] A possible explanation is that perhaps the enzyme became sensitive to an increase in the duration of exposure to heat rather than to an increase in temperature.[22] The activation energies, E_a, for the free and immobilized cellulase and pectinase were obtained from the slope of the Arrhenius plot of In v_o against $\frac{1}{T}$. The E_a values obtained in this study were very low and this might indicate that very few energy were needed to form the transition state complex. This suggests that both cellulase and pectinases in free and immobilized forms had higher hydrolytic capacity. The immobilized forms of the cellulase and pectinase had the higher E_a values of 6.80 kJ/mol/K (R^2 = 0.997) and 7.2 kJ/mol/K (R^2 = 0.995), respectively, which was way higher than that of the free enzymes which had E_a values of 5.42 kJ/mol/K (R^2 = 0.975) and 5.42 kJ/mol/K (R^2 = 0.991), respectively, (Fig. 9; the Arrhenius plot for immobilized cellulase and pectinase not shown). A possible explanation is that perhaps the cellulase and pectinase needed to develop an effective

Table 3. Thermodynamic properties of free and immobilized cellulase and pectinase from *Aureobasidium pullulans* NAC8.

T (K)	E_d^* (kJ/mol)	R^2	ΔH_d^* (kJ/mol)	ΔG_d^* (kJ/mol)	ΔS_d^* (J/mol/K)	Overall ΔS_d^* (J/mol/K)	Overall ΔH_d^* (kJ/mol)
Immobilized cellulase							
313	37.5	0.995	34.9	91.9	−182.1	183.4	34.7
323			34.8	94.0	−183.3		
333			34.7	95.8	−183.4		
343			34.6	97.5	−183.4		
Free cellulase	33.7	0.983					
313			31.1	91.2	−192.0	190.5	31.0
323			31.0	92.8	−191.3		
333			30.9	94.6	−191.3		
343			30.8	97.0	−193.0		
Immobilized pectinase							
313	44.7	0.945	42.1	93.0	−162.6	168.1	43.4
323			42.0	94.9	−163.7		
333			41.9	96.8	−164.9		
343			41.8	96.4	−159.2		
Free pectinase							
313	29.8	0.968	27.2	88.1	−194.6	196.1	26.0
323			27.1	90.0	−194.7		
333			27.0	92.4	−196.4		
343			26.9	93.9	−195.3		

conformational structure in order to form a well-oriented and specific complex with the substrate.[21] A unique thermodynamic property used in distinguishing if catalyzed reactions are solely controlled by temperature only or by other extraneous factors is the temperature quotient (Q_{10}). The Q_{10} values varied between 1 and 2 for most enzyme catalyzed reaction which its totally dependent on temperature. Values outside this range signify that other extraneous factors other than temperature controlled the rate of reaction. Table 1.

The Q_{10} values obtained for the free and immobilized cellulase and pectinase (values ranged between 1 and 1.2) meant that temperature was influential on the reaction rate. A similar observation was observed for *A. aculeatus* free and immobilized pectinase.[18]

Thermodynamic parameters for enzyme thermal denaturation

Table 3 details the several thermodynamic parameters obtained for the free and immobilized cellulase and pectinase. The activation energy of denaturation, E_d^*, directly related to the ΔH_d^* (activation enthalpy of deactivation) and was determined from the semi-log plots of In k_d vs. $^1/_T$. Large positive values of both E_d^* and ΔH_d^* are indicative of high enzyme stability. The E_d^* obtained for immobilized and free cellulase are 37.5 kJ/mol ($R^2 = 0.995$) and 33.7 kJ/mol ($R^2 = 0.983$), respectively, while the immobilized and free pectinase had E_d^* values of 44.7 kJ/mol ($R^2 = 0.945$) and 29.8 kJ/mol ($R^2 = 0.968$), respectively. The immobilized cellulase and pectinase had higher E_d^* values than the free enzymes, and this is interpreted as the latter having more resistivity to thermal inactivation compared to the free enzyme.[20] ΔH_d^*, also known as the activation enthalpy of thermal denaturation, is used in quantifying the overall amount of energy required for enzyme thermal denaturation. This is because thermal denaturation of enzymes occurs as a result of alteration in non-covalent linkages/hydrophobic interactions. This generally leads to increased

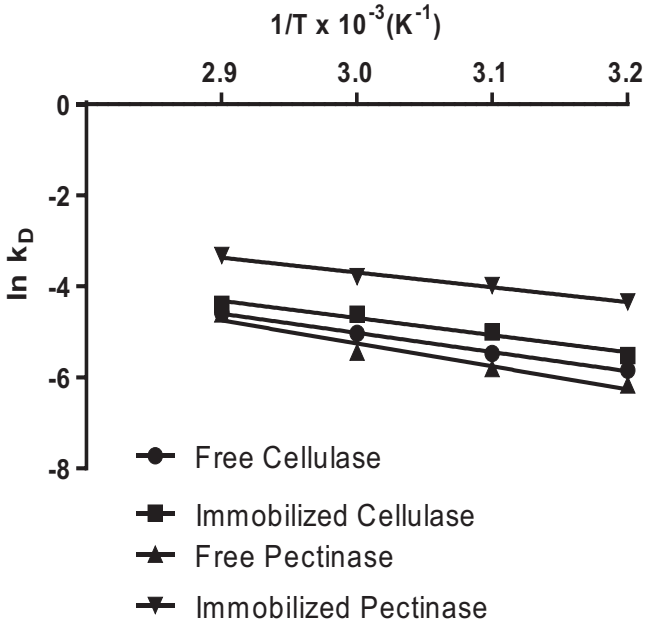

Figure 10. Arrhenius plot to estimate the thermodynamic parameters of thermal denaturation of free and immobilized cellulase and pectinase.

enthalpy. The ΔH_d^* values obtained for the immobilized enzymes (34.6–34.9 kJ/mol and 41.8–42.1 kJ/mol for cellulase and pectinase, respectively) were higher than the free enzymes (30.8–31.1 kJ/mol and 27.2–26.9 kJ/mol for cellulase and pectinase, respectively) (Table 3), and it can be concluded that immobilization increased the energy needed to thermally denature the enzymes as well as provide the necessary stability required.[43] The values obtained were in agreement with the overall ΔH_d^* values obtained from the plot of In $\frac{k_d}{T}$ against $^1/_T$ (Fig. 10 and Table 3). Also, since the energy required for the removal of $-CH_2$ moiety from a hydrophobic bond is approximately 5.4 kJ/mol,[20] we can estimate the formation of the transition state leading to the thermal deactivation of immobilized and free cellulase and pectinase implied the disruption as an average of 6.5, 5.8, 7.8, and 5.0 non-covalent bonds, respectively.

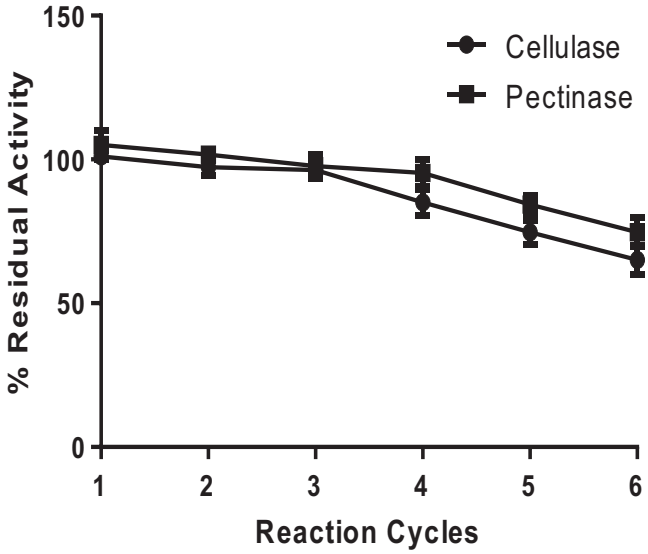

Figure 11: Catalysis by the immobilized cellulase and pectinase over six reaction cycles. The immobilized enzymes were used for repeated hydrolysis of carboxymethyl cellulose and pectin. At the end of each reaction cycle, the immobilized enzyme was washed in 0.5 M acetate buffer pH 5.5 and then used for another round of catalysis. Cellulase and pectinase activities were routinely monitored. Error bars represent standard deviation ($n = 3$).

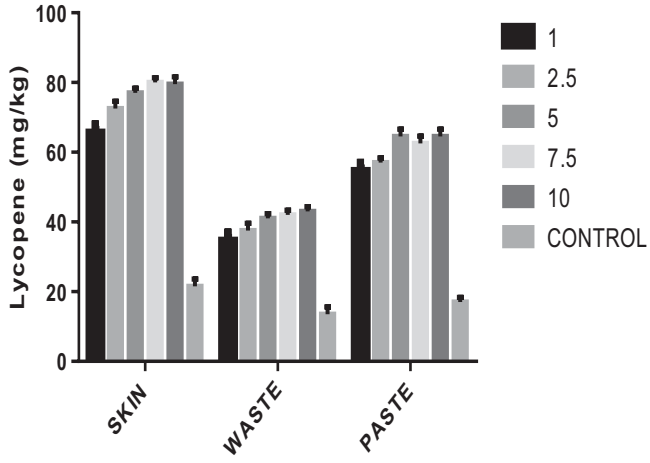

Figure 12. Determination of lycopene content using immobilized cell wall degrading enzymes by varying the amount (g) of immobilized enzyme.

The disruption of the native enzyme structure is usually accompanied by an increase in the degree of disorderliness or activation entropy (positive entropic value ΔS_d^*).[44] In this study, negative values were obtained for ΔS_d^*, i.e., $-183.4 \leq \Delta S_d^* \leq -182.1$ and $-162.6 \leq \Delta S_d^* \leq -159.2$ for immobilized cellulase and pectinase respectively while the free cellulase and pectinase had activation entropy of $-193.0 \leq \Delta S_d^* \leq -192.0$ and $-195.3 \leq \Delta S_d^* \leq -194.6$, respectively. The values obtained were in agreement with the overall ΔS_d^* values obtained from the plot of $In\frac{k_d}{T}$ against $^1/_T$ (Fig. 10 and Table 3). These values for ΔS_d^* are indicative of increased orderliness of the activated complex as well as its increased thermostability from 40 to 70 °C. The changes observed with the ΔS_d^* relative to the temperature might be as a result of the effects of conformational changes and thermal flux on the enzyme.[42] From the changes observed in the ΔS_d^* *and* ΔE_d^* due to immobilization, a conclusion that the hydrophobic core of the immobilized forms of cellulase and pectinase decreased as the thermostability increased. The data obtained in this study for the ΔH_d^*, ΔS_d^*, *and* ΔE_d^* for immobilized cellulase and pectinase were in good agreement with the observations immobilized pectinase.[18]

The Gibbs free energy, ΔG_d^*, is often regarded as one of the most important thermodynamic parameters due to the fact that it inculcates both enthalpic and entropic considerations. Hence, it is a more accurate tool for elucidating and estimating the overall stability of enzymes.[45] To understand the significance of ΔG_d^*, it is known that negative values indicate a sudden process of thermal inactivation while positive values are synonymous with increased resistance to thermal denaturation. In this study, the ΔG_d^* values obtained in this study were all positive values for all temperatures considered, the ΔG_d^* values were also very close to each other, i.e.,$91.9 \leq \Delta G_d^* \leq 97.5$ and $93.0 \leq \Delta G_d^* \leq 96.4$ for immobilized cellulase and pectinase respectively while the free cellulase and pectinase had ΔG_d^* values of $91.2 \leq \Delta G_d^* \leq 97.0$ and $88.1 \leq \Delta G_d^* \leq 93.9$. This is suggestive of a very thermostable immobilized and free cellulase and pectinase which is due to the greater energy that allowed the enzyme to prevent unfolding of its activated complex.[37] It can be summarized that all the kinetic and thermodynamic properties considered for both free and immobilized cellulase and pectinase indicate a thermostable enzyme.

Reusability of the immobilized cell-wall degrading enzymes

After immobilization by entrapment of the *CWDE* on alginate, there was a yield of about 59% and 72% for cellulase and pectinase respectively after the sixth cycle of catalysis (Fig. 11). The progressive loss in enzymatic activities after might be due to the leakage due to the repeated washing.[36] In this study, the immobilized enzyme retained 100% of its starting activity after the first three cycles of catalysis this was more than the 80% observed for immobilized pectinase from *A. aculeatus*.[18]

Lycopene release from tomato skin, waste, and paste using immobilized cellulase and pectinase

The immobilized enzyme was applied for the repeated extraction of lycopene from tomato tissues due to the ease of recycling associated with the immobilized enzymes compared with the free enzyme. The tomato tissues contain cellulose and pectin which trap the lycopene; hence, the immobilized enzyme broke down the cellulose and pectin component present in the tomato for efficient lycopene recovery from them. From this study, the maximum lycopene concentration was obtained from the tomato skin (80 mg/kg) (Fig. 12). The tomato waste had the least amount of lycopene (42 mg/kg). The values obtained for lycopene from paste (60 mg/kg) might vary based on the effect of processing and storage on the lycopene content or the cultivar used in its manufacturing.[46] It can be assumed that due to the breakdown of the tomato tissues for increased lycopene

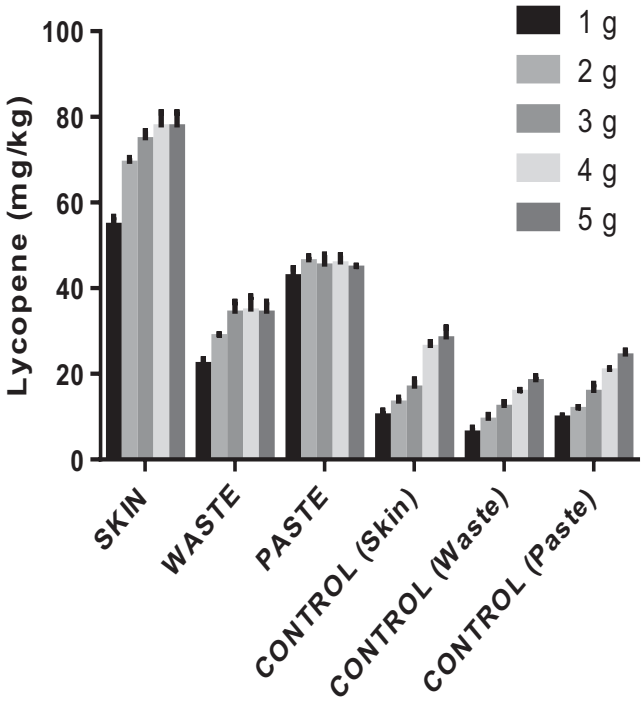

Figure 13. Determination of lycopene content using immobilized cell wall degrading enzymes by varying the mass of the skin waste and paste.

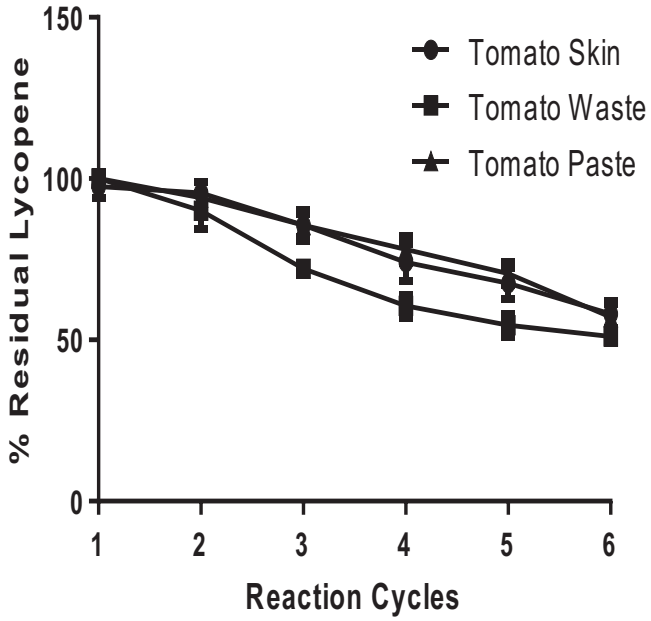

Figure 14. Reusability of the immobilized enzyme in lycopene extraction from tomato skin, waste, and paste. Error bars represent standard deviation ($n = 3$).

recovery, cellulolytic and pectinolytic enzymatic activities occurred. It was observed that increasing the concentration of the tomato skin, waste, and paste (*swp*) resulted in the gradual increase in the amount of lycopene but no significant difference ($p < 0.05$) was observed when the concentration was increased from 3 to 5 g (Fig. 12). This might be due to the fact that the amount of immobilized enzyme (1 g) used for the breakdown of the cellulose and pectin component was not enough to further break them down thus facilitating the eventual release of more lycopene entrapped in the tomato tissues or tomato paste as the case may be. Zuorro and Lavecchia[38] reported that the lycopene yield obtained from tomato paste and peel were 35 mg/100 g and 350 mg/100 g, respectively, while Rahimpour et al.[10] reported a maximum lycopene concentration of 94.3 mg/kg from tomato wastes. In our study, the maximum lycopene extracted was from the tomato skin with a value of 80 mg/kg compared to 42 mg/kg and 60 mg/kg for tomato waste and paste respectively (Fig. 12). The values obtained by Choudhari and Antananyarana[3] for lycopene from fruit pulp wastes using cellulase and pectinase were 132 mg/kg and 108 mg/kg, respectively. The variation observed in these studies might be explained by the fact that tomatoes contain different amounts of the polysaccharides in its different parts. Several factors might bring about these differences and namely the ripening stage as well as the variety of the tomatoes.[6] Attempts were made to further facilitate the breakdown of the cellulose and pectin component of the tomato *swp* by increasing the amount of enzyme and it was observed that a significant increase in the lycopene content was correlated with the increased enzyme load (Fig. 13) but at a 7.5 g of the immobilized enzyme, no significant increase in lycopene content was observed. This observation was in agreement with the report of Rahimpouri and Dinani[10] where it was reported that a significant increase in the enzyme concentration did not lead to increased lycopene release from the tomato tissues. Chouudhari and Anathananarayan[3] even posited that the decreased lycopene at increased enzyme load might be due to the quick hydrolysis by the enzyme and presumably end-product inhibition of the enzyme. The immobilized enzyme was able to continuously extract lycopene over six cycles and a lycopene yield of 43% was obtained (Fig. 14). This is quite remarkable compared to the report of Ladole et al.[16] where the enzyme co-immobilized magnetic nanoparticles combined with ultrasound was reused for six cycles and it could only recover about 50% of the lycopene.

Characterization of the lycopene

From the FTIR spectra (Fig. 15), the presence of characteristic vibrational peaks which represent the unique fingerprints of lycopene were observed. For example, a peak was observed around 874.4 cm^{-1} in all the spectra, this peak corresponds to the stretching vibration of the C–C–H_2 and C–OH groups found in lycopene. A characteristic peak observed at 1508.3 cm^{-1} confirms the deformation vibration of –CH_2 present in the lycopene molecule. Other peaks found at 1174.3 and 1271.3 cm^{-1} corresponds to the asymmetric stretching of C–H groups unique to lycopene. The FTIR spectra data obtained for lycopene corresponds to the observations of Rahimpour and Dinani[10] and Ladole et al.[16]

Conclusion

In conclusion, cell-wall degrading enzymes such as cellulase, pectinase, amylase, and cellulase were produced by induction

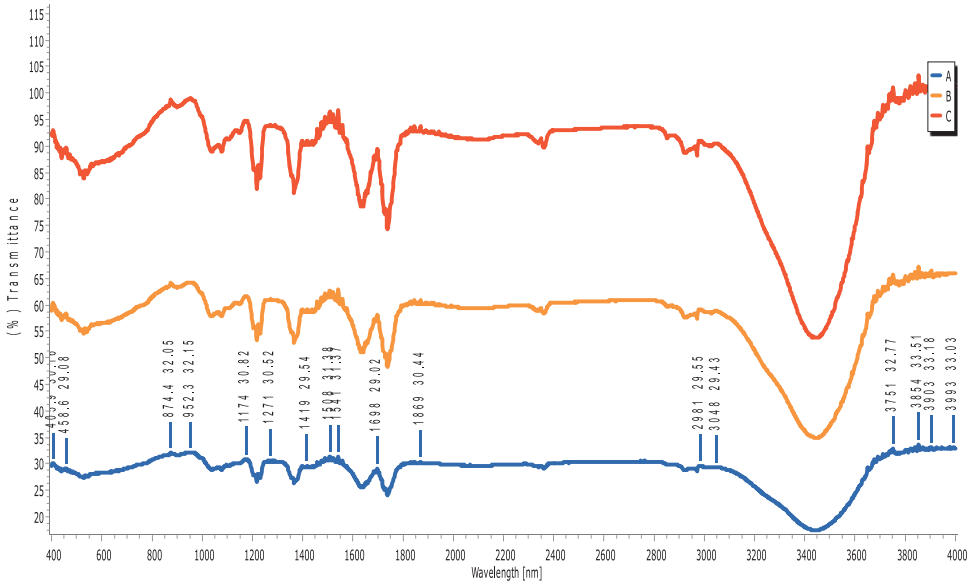

Figure 15. Fourier transform infrared spectra of lycopene from tomato skin extracted using (A) immobilized enzyme (B) free enzyme (C) solvents only.

of *A. pullulans* using orange peel waste. Orange peels contain pectin and cellulose which makes them suitable, cheap, and readily available substrates for the production of useful hydrolytic enzymes. From the kinetic and thermodynamic parameters obtained from the thermal inactivation studies, it was concluded that both the free and immobilized cellulase and pectinase were thermostable at 40–50 °C. Also, the immobilized enzymes retained more than 59% and 72% (for cellulase and pectinase, respectively) of the starting activity after six catalytic cycles; this makes it suitable for industrial exploitation especially in the food industry. Both the free and immobilized cellulase and pectinases were efficient in the extraction of lycopene from tomato waste and paste, but the enzyme entrapped in alginate had higher lycopene recovery.

Disclosure statement

No potential conflict of interest was reported by the authors.

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