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Optimal conditions for pectinase production by S*accharomyces cerevisiae (*ATCC 52712) in solid state fermentation and its efficacy in orange juice extraction

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Abstract:*Pectinases are the group of enzymes that degrade pectin and one of the enzymes widely used in the food industry. In the fruit juice industry, pectinases are often used to enhance juice extraction and clarification. This paper reports on the optimal conditions for pectinase production by Saccharomyces cerevisiae and preliminary evaluation of its efficacy in orange juice extraction. Fermentation was carried out on corn cob/orange peel substrate mixture. The crude enzyme wasextracted from the solid medium using different solvent systems after which its usefulness in the extraction of orange juice was evaluated.The optimal conditions established for the enzyme production were; 6 days of fermentation using a ratio of 80:20 percent corn cob and orange peels as substrate, pH 4.0 at 30^{o}C with an inoculum size of 10.46 x 10^{6} cells/ml. Maximum enzyme extraction was achieved using 0.1M NaCl when the fermented mash was agitated for 30 min during enzyme extraction. Enzyme activity was improved by ammonium sulphate saturation (60%). An enzyme dosage of 0.02% (40mg protein/200g orange mash) at 45min reaction time led to an increase in juice extraction by 123.4% compared to controls. This study thus demonstrated the possibility of local enzyme production for application by local industries in Ghana.*

Keywords: *Corncob, Orange juice, Orange peel, Pectinase,Saccharomycescerevisiae,*

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I. Introduction

It is estimated that, nearly 20-40% of fruits produced in developing countries are lost due to spoilage, mishandling during transportation and inadequate cold storage facilities and processing techniques, hence the need for conversion into juice concentrate [1]. For effective juice extraction, enzymes such as pectinases provide the best option since mechanical pressing of pectin-rich fruits leads to low yieldsand gives juice that remains bound to the pulp to form a jellified mass [2]. In order to improve juice extraction, there is the need to degrade pectin using pectinases [3].

Production of pectinases constitutes about 10 % of the overall industrial enzyme preparations, they are widely used in the food industry for juice, and wine production [4] .Commercial pectin enzymes have been employed in starch extraction from sweet potato [5], yam [6] and cassava [7]. Some conventional industrial processes over the years, such as plant fiber processing, tea and coffee industries, treatment of industrial wastewater containing pectinaceous material and paper manufacturing have used pectinases at a point in their production processes [8].Our laboratory has been investigating the production of pectin enzymes from *Saccharomyces cerevisiae* ATCC 52712 and its application on pectin rich fruits [9, 10, 11, 12, 13]. Successful use of the yeast enzyme in enhancing pineapple juice extraction leading to increase in juice yield with significant reduction in processing time as compared to controls without enzyme application has been reported [14].Ghana has a number of small scale fruit juice processors who can benefit from pectin enzyme applications if they have access to the enzymes locally.

The objective of this study was to optimize conditions for pectinase production by *Saccharomyces cerevisiae* using solid-state fermentation and conduct a preliminary assessment on its efficacy in orange juice extraction.

II. Materials And Methods

Saccharomyces cerevisiae ATCC 52712 was purchased from the American Type Culture Collection, MD, USA. The corncobs and orange peels used were obtained from local sources in the Kumasi metropolis, Ghana. The uniformly ripened, but firm oranges for juice extraction studies were obtained from Kumasi Central market. All other chemicals, which are of analytical grade, were obtained from Sigma – Aldrich, St. Louis, MO, USA.

2.1 Methodology

Yeast cells, obtained in a freeze-dried form were revived and propagated in a yeast extract/peptone/dextrose agar medium.

2.2 Pectinase production

Pectinase production was carried out by solid-state fermentation (SSF) using different combinations of corncobs and orange peels. Enzyme production was optimized for the following parameters: fermentation time, orange peel to corncob ratio, temperature, pH and inoculum size. After enzyme production, its extraction from the solid substrate was also optimized with respect to best solvent and agitation period for extraction. The optimal conditions established for enzyme production were then used simultaneously to produce the enzyme on a larger scale for use in juice extraction.

After $(NH_4)_2SO_4$ concentration to improve the enzyme activity, its effect on orange juice extraction with regards to reaction time and enzyme dosage were investigated.

The entire methodology is shown in the flow chart below:

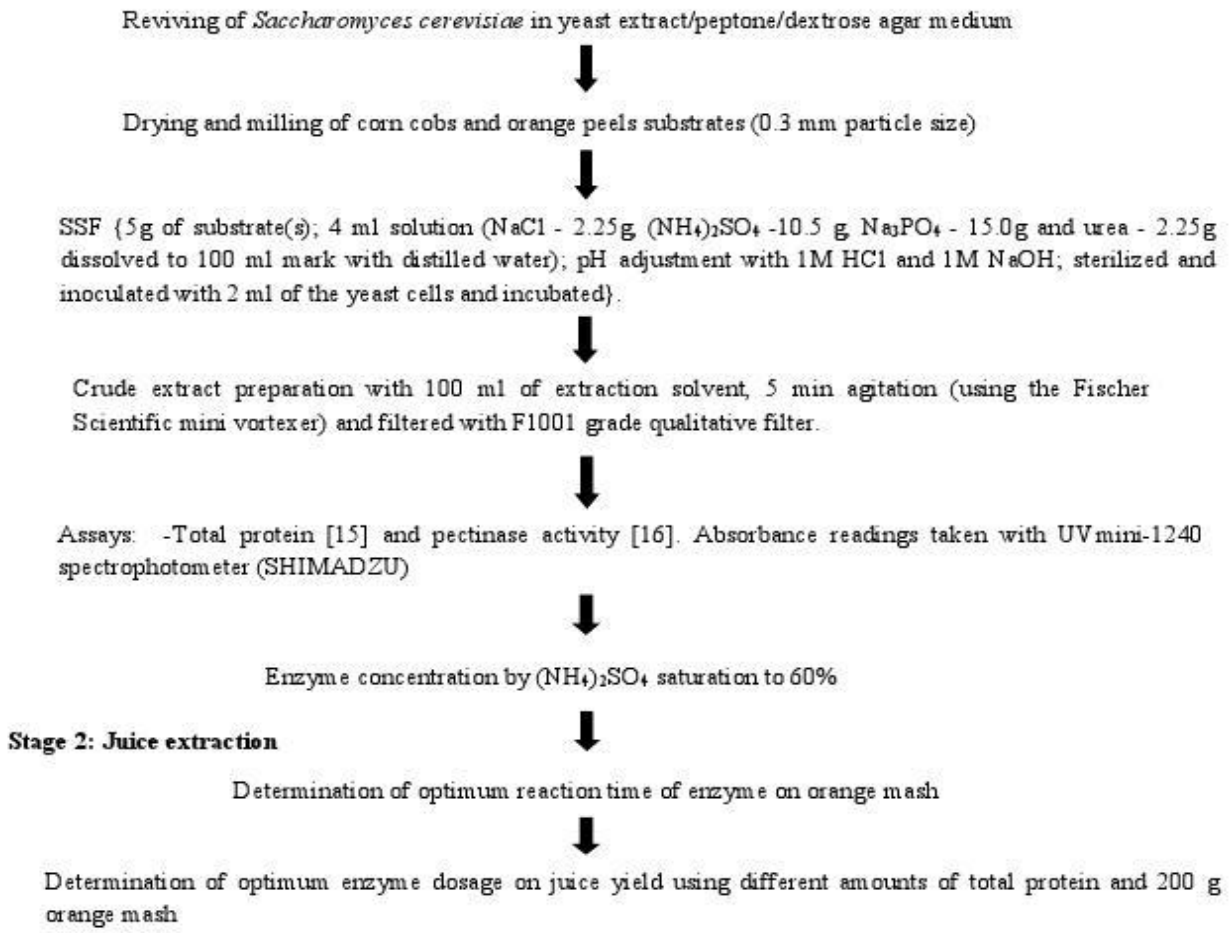

Fig. 1. Flow chart of enzyme production and application in orange juice extraction.

NB: 1*. The turbidity of the yeast culture measured as absorbance and correlated to the total number of yeast cells (www.pangloss.com/seidel/Protocols?ODvsCells.html accessed 20/09/2013) was used to determine for inoculum concentration.*

2*. The solvents used for crude enzyme extraction were selected after perusal of literature*[17, 18, 1].

2.3 Determination of pectinase activity

Pectinase activity was measured by a slight modification of the 55th Joint Expert Committee for Food Additives- JECFAmethod [16].This assay is based on the hydrolysis of pectin and the resulting galacturonic acid. The reaction mixture consisted of 0.1 ml of the enzyme solution and 3.0 ml 0f 0.5% pectin solution. The mixture was incubated for 5 min at 30°C. One (1) ml from the above resultant solution was added to 10 ml of acetate buffer (pH 5.0) and mixed thoroughly after which the absorbance at 235 nm was read over 8 min at one min interval using the appropriate buffer as blank. One unit of pectinase activity causes an increase of 0.010 of absorbance per minute under the assay conditions (pectin 0.5%, pH 5.0 at 30°C). Determinations were done in triplicate.

2.4 Effect of ammonium sulphate saturation on protein concentration

Precipitation of the crude enzyme was carried out using ammonium sulphate $\{(NH_4)_2SO_4\}$. The extracted enzyme solution was saturated with ammonium sulphate up to 80%. Precipitates were obtained by gently stirring the mixture and leaving on ice for 30 min after which the mixture was centrifuged at 3600 g for 15 min. The resultant precipitates were assayed for protein concentration [15] and pectinase activity [16].

2.5 Effect of enzyme reaction/holding time on volume of orange juice extracted

Two-hundred (200) grams mashed oranges were weighed into labelled beakers and 1 ml of the concentrated enzyme extract (10 mg protein /ml) was added to each beaker, stirred and covered with plastic wraps and left to stand for various reaction times. At the end of each reaction time, the contents were filtered over 6 min period using a funnel and Whatman No. 1 filter paper and the volumes of juice obtained were compared to the control (orange mash with no enzyme).

2.6 Enzyme dosage effect on volume of juice produced

Different volumes (1-5 ml) of the concentrated enzyme extract containing approximately 10 mg/ml protein were added to 200 g orange mash to obtain different dosages ranging from 10 – 50 mg total protein per 200 g mash. These were allowed to stand for the optimum reaction time established earlier after which each was filtered and the volume of juice obtained measured. The control used was orange mash with equal volume of solvent in place of enzyme.

III. Results And Discussion

Figures 2 to 8 and table 1 shows the optimal parameters for pectinase production while Fig. 9 to 11 shows the optimal parameters studied for effective orange juice extraction.Enzymes function best at optimal conditions, making optimization of conditions for effective pectinase activity important. It is therefore, important to consider the effect of these conditions in detail.

3.1 Effect of fermentation time on pectinase activity on corncob substrate only

The effect of duration of fermentation on pectinase activity with corncobs as substrate is illustrated in Fig. 2. Optimum specific activityof pectinase was recorded on the 6th day of fermentation (Fig. 2), while there was an increase in protein concentration from day 3 to 15 in both first and second filtrates (Fig. 2).

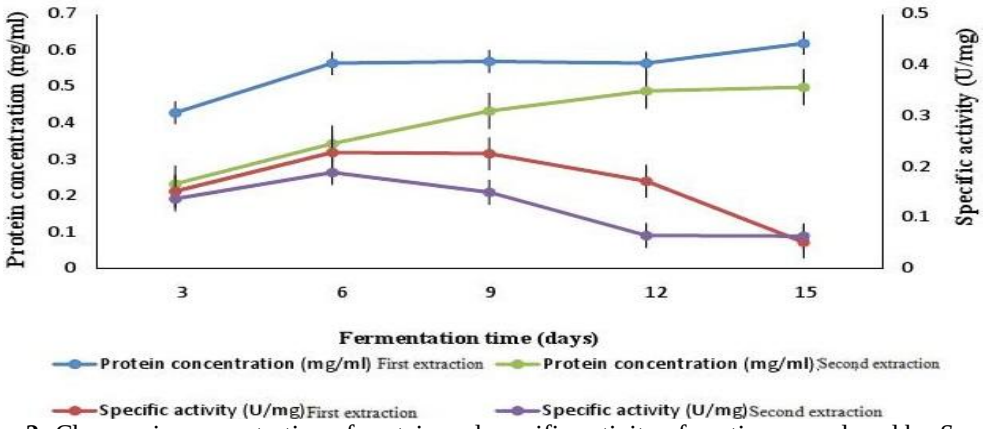

Figure 2: Changes in concentration of protein and specific activity of pectinase produced by *S. cerevisiae* with fermentation time. *Fermentation conditions -100% corncobs, pH 4.5 at 30 °C with acetate buffer (pH 5.0) as extraction solvent.

The decrease in activity of pectinase beyond the 6th day of fermentation could be attributed to catabolite repression [19] and lower content of pectin in the medium for pectinase production [20].Although the protein concentration was highest on the 15th day of fermentation, the optimum specific activity of pectinase was achieved on the 6th day. This therefore indicates that other proteins aside pectinases such as xylanases [21, 22, 23, 24]and cellulases [25, 26, 27]could be present in the crude extract, since the cell wall of plants is composed primarily of polysaccharides (such as cellulose, hemi cellulose and pectin substances) [28].

3.2 Effect of supplementation of corncobs with different proportions of orange peels on pectinase production

Various formulations of corncobs supplemented with orange peels on pectinase production showed that, a ratio of 80%**:**20% gave optimum results (Fig. 3). Whereas protein concentration increased with increase in proportion of orange peels, enzyme activity peaked at 80:20%. The combination of 80:20% of corn cobs to orange peels might have favoured adequate oxygen supply by increasing inter-particle spacing thereby enhancing growth of the yeasts, pectinase yield and easier solvent penetration during enzyme extraction [29, 30, 31], leading to significant differences ($p<0.05$) obtained in pectinase activities and protein concentrations between first and second extractions (Fig. 3). The citrus peels, serving as an inducer for pectinase production has also been reported [31].

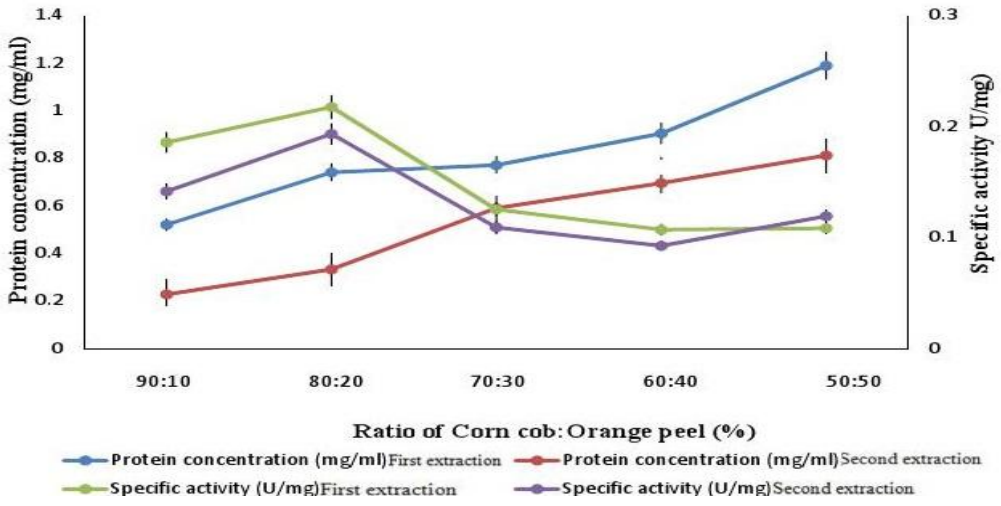

Figure 3. Changes in protein concentration and specific activity of pectinase during fermentation of different proportions of corncobs and orange peels by *S. cerevisiae*.

*Fermentation conditions -6 d of fermentation, pH 4.5 at 30^{o}C with acetate buffer (pH 5.0) as extraction solvent.

3.3 Effect of pH on pectinase production and activity

The effect of pH on pectinase production and activity is shown in Fig. 4. Optimum pectinase activity (0.24 U/mg) was observed at pH 4.0 (Fig. 4). pH alters enzyme conformation, recognition site, active site and substrate conformation [32], hence determining its optimum value is very critical in biochemical characterization of enzymes. The decline in activity beyond pH of 4 (Fig. 4) may be due to denaturation of the enzyme which is a common phenomenon during fermentation due to the release of various by-products in the media [20] or instability of the enzyme at extreme pH values since they are proteins that are generally denatured at extreme pH values [33].Pectolytic enzymes from *Saccharomyces cerevisiae* (ATTC 52712) have been found to be active at broad pH ranges of 3.5-5.0 [14].

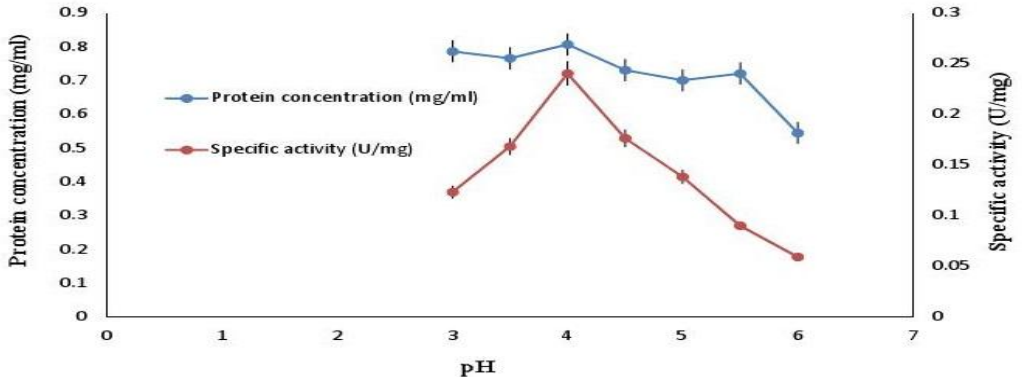

Figure 4. Effect of pH on protein concentration and specific activity of pectinase during fermentation with *S. cerevisiae.*

*Fermentation conditions -6 dof fermentation, 80% corncob: 20% orange peel, pH 4.5 at 30^{o}C with acetate buffer (pH 5.0) as extraction solvent.

3.4 Effect of temperature on pectinase activity

Temperature's effect on pectinase activity is shown in Fig. 5. The optimum pectinase activitywas found to be at 30^{o}C after which there was a decline. The reduction in enzyme activity and protein concentration beyond 30^{o}C (Fig. 5) during fermentation at elevated temperatures may be due to unfavourable heat stress encountered by the yeast cells; this excess heat effect possibly changed the physical properties of the organism's cell membrane thereby affecting protein secretion and uncoiling of some of the secreted proteins into random configurations due to heat stress, leading to decline in protein concentrations and pectinase activities [34].

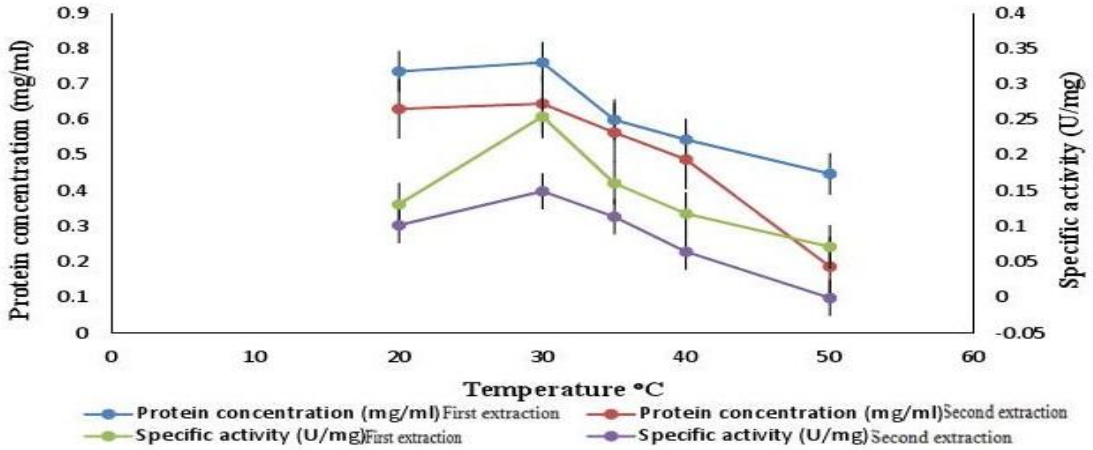

Figure 5. Effect of temperature on protein concentration and specific activity of pectinase during fermentation with *S. cerevisiae*.

*Fermentation conditions -6 d of fermentation, 80% corncob: 20% orange peel, pH 4.0 at 30^{o}C with acetate buffer (pH 5.0) as extraction solvent.

3.5 Effect of inoculum size on pectinase production and activity

With respect to cell density of *Saccharomyces cerevisiae* on enzyme activity, 10.46 x 10^{6} cells/ml was the optimum inoculum size for pectinase production (Fig. 6) indicating that this cell concentration was sufficient in colonizing the substrate particles for optimum enzyme activity to be obtained [35].

Reduction in activity with further increase in cell concentration beyond the optimum level might be due to clumping of cells, thereby reducing carbon and oxygen uptake leading to reduction in pectin enzyme released [36].

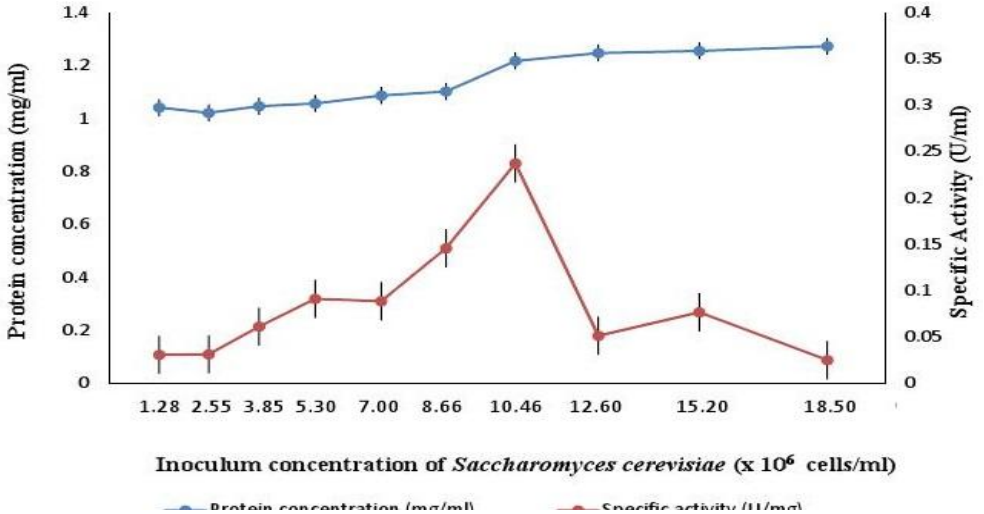

Figure 6. Effect of inoculum concentration on protein concentration and specific activity of pectinase during fermentation with *S. cerevisiae*.
*Fermentation conditions -6 d of fermentation, 80% corncob: 20% orange peel, pH 4.0 at 30^{o}C with acetate buffer (pH 5.0) as extraction solvent.

3.6 Effect of agitation time during enzyme extraction on pectinase activity

The effect of period of agitation during enzyme extraction from the solid medium after fermentation gave 30 min as optimum time. Though some reports however indicated that degrading ability of the enzyme is enhanced by agitation [34, 37] the results obtained in this study showed a decline after 30 min of agitation. This loss of activity beyond 30 min could be attributed to the fact that when a higher level of mechanical agitation was introduced into the system, the level of surface modification of the enzyme increased, thereby decreasing its activity [38].

3.7 Effect of extraction solvent on pectinase activity

The effect of the extraction solvents is shown in Fig. 8. The highest pectinase activity (0.296 U/mg) was found with NaCl (0.1M). Adsorption of enzymes to cells or solid substrates have been attributed to ionic bond, hydrogen bond and Van *der* Waal's forces [1].Extracted enzyme by NaCl (0.1M) was significantly higher ($p<0.05$) than the other two solvents.This therefore makes extraction an area worth considering in the recovery of enzyme from fermented biomass; hence, selection of a suitable solvent is necessary. The action of these solvents helped break the bonds between carbohydrates and proteins [39].NaCl is a much cheaper salt compared to acetate and citrate buffers and thus can be considered an advantage in this study.

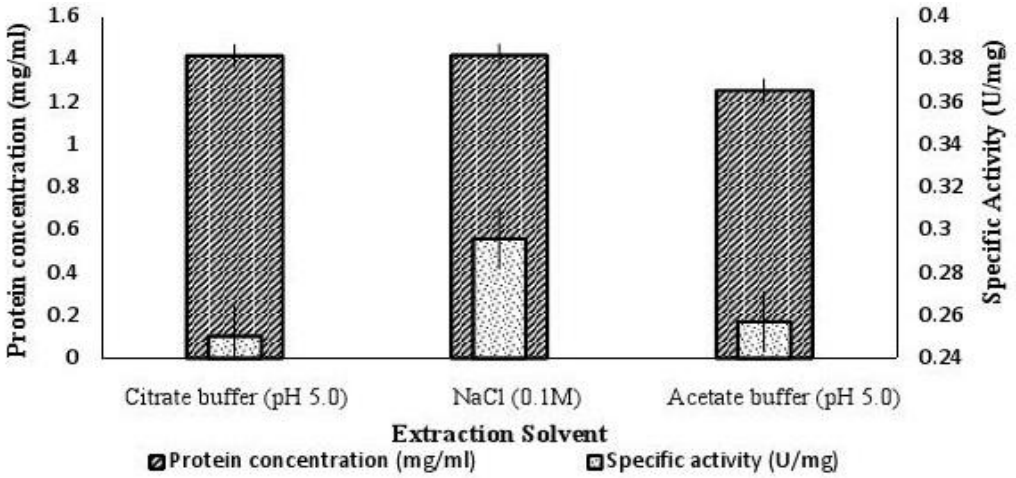

Figure 8. Effect of extraction solvent on protein concentration and specific activity of pectinase from the solid medium. *Fermentation conditions -6 d of fermentation, 80% corncob: 20% orange peel, pH 4.0 at 30^{o}C, 30 min agitation, 10.46 x 10^{6} cells/ml.

3.8 Pectinase production under the established optimized conditions

When all the optimal parameters were combined to produce the pectinase enzyme, a total activity of 29.57 U and specific activity of 0.296 U/mg were obtained as shown in table 1.

Table 1: Activity of pectinase produced using the optimal conditions previously established

Pectinase activity (U/ml)	Protein concentration (mg/ml)	Total activity (U)	Specific activity of pectinase (U/mg)
0.3548	1.2000	29.57	0.2957

Pectinase production under the established optimal conditions showed 35.89% increase in activity over corncobs supplemented with orange peels alone. This increase further shows the need for optimization of fermentation parameters during fermentation for increase in enzyme activity.

3.9 Application of pectinase produced in orange juice extraction

The enzyme produced under the established optimal conditions was further concentrated by ammonium sulphate precipitation to increase its activity prior to its use in juice extraction.

3.9.1 Effect of $(NH_4)_2SO_4$ precipitation on pectinase activity

Table 2 shows the various concentrations of the ammonium sulphate that were employed to precipitate the crude enzyme. The pectinase activity progressively increased from 20% $(NH_4)_2SO_4$ (0.29 U/mg)concentration to an optimum concentration of 60% $(NH_4)_2SO_4$, where the activity was highest (0.43 U/mg). As the first step in many purification processes, ammonium sulphate precipitation has shown to be a quick way of eliminating some contaminant proteins, thus, increasing the activity of the enzyme. The ability of the $(NH_4)_2SO_4$ to concentrate enzymes makes it employable even in later stages of purification to concentrate enzymes from dilute solutions.

Table 2: Partial purification table for the extracted crude pectinase from *S. cerevisiae*(ATCC 52712)

Purification step	Total activity (U/ml) X 10 DF	Total protein (mg/ml)	Specific activity (U/mg)
Crude extract	0.35	1.20	0.29
20% $(NH_4)_2SO_4$	1.39	4.10	0.34
60% $(NH_4)_2SO_4$	3.97	9.50	0.43
80% $(NH_4)_2SO_4$	4.16	11.10	0.37

3.9.2 Effect of enzyme reaction/holding time on volume of juice extracted

Holding or reaction time was undertaken to determine the optimum time for enzyme interaction with the orange mash substrate for effective extraction of juice. From Figure 10, 45 min gave the optimum reaction time at which the highest volume of free-run juice (8.5 ml) was obtained beyond which no significant increase($p>0.05$) in juice volume extracted was obtained. Free-run juice as used here is simply the juice produced at the end of reaction and filtered (with Whatmann No. 1 filter paper -for 6 min) with no external applied pressure [11].

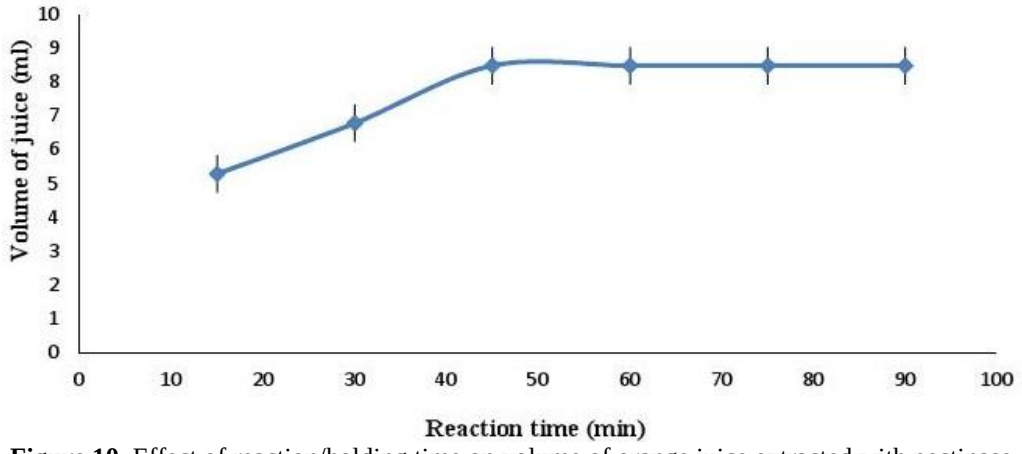

Figure 10. Effect of reaction/holding time on volume of orange juice extracted with pectinase.

*Fermentation conditions- 6 d fermentation, 80% corn cob : 20% orange peel, pH 4.0 at 30^oC, 30 min agitation, 10.46 x10^6cells/ml , 0.1M NaCl as solvent for extraction, 60%$(NH_4)_2SO_4$ protein saturation and 10 mg of the enzyme produced per 200 g orange mash.

3.9.3 Effect of enzyme dosage on the volume of free-run juice collected

Figure 11 illustrates the effect of enzyme dosage on volume of juice produced. The optimum enzyme dosage for best juice extraction was 4 ml of 10 mg/ml (40 mg) total protein per 200 g of orange mash (0.02% enzyme dosage). Forty mg total protein per 200 g of orange mash resulted in 123.4 % increase in orange juice extracted while 10 mg total protein per 200 g of orange mash led to 15 % increase in free-run juice over control. Therefore, the application of this enzyme enhanced hydrolysis of pectic substances in the mash thereby resulting in the release of more juice.

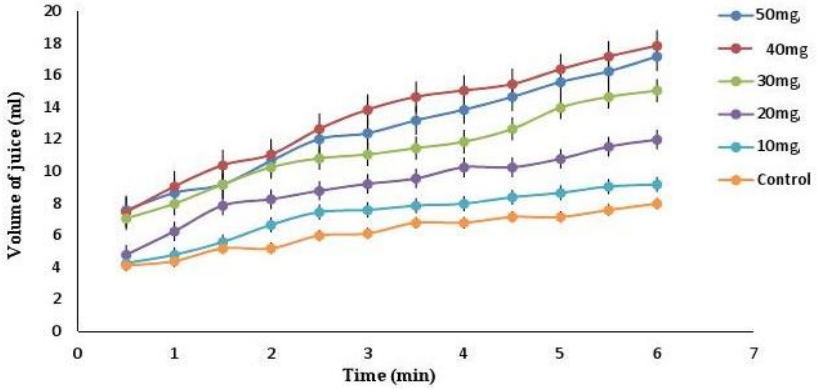

Figure 11. Effect of enzyme dosage on the volume of juice produced.
*Fermentation conditions- 6 d of fermentation, 80% corn cobs: 20% orange peels, pH 4.0 at 30^{o}C, 30 minagitation, 10.46 x10^{6}cells/ml, 0.1M NaCl as solvent for extraction, 60%$(NH_4)_2SO_4$ protein precipitation and 45 min reaction time.

However, at a higher enzyme concentration (50 mg total protein/200g of orange mash) a decline was obtained. The medium could have been saturated with the enzyme and the increasing amount of products formed could have reached inhibitory concentrations resulting in decrease in enzyme activity [2].This probably accounted for the decrease in orange juice extracted with increase in enzyme dosage above 0.02 %.

IV. Conclusion

The feasibility of using microorganisms to locally produce industrially competent pectinases has been established in this study. The enzyme utilization by local industries could prove to be convenient and cost effective since 40 mg total protein per 200 g of orange mash could efficiently extract 123.4% more orange juice with lesser reaction time of 45 min than the extraction without enzymes. This will tend to reduce wastage of fruits and a boost to the local juice manufacturers, especially when this enzyme is produced on a large scale.

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