Optimising growth conditions for the pectinolytic activity of *Kluyveromyces wickerhamii* by using response surface methodology

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Abstract

This present study was undertaken to find optimum conditions of pH, temperature and, period of incubation for the pectinolytic activity of *Kluyveromyces wickerhamii* isolated from rotting fruits and to assess the effect of these factors by use of response surface methodology (RSM). A central composite rotatable design was used as an experimental design for the analysis of the allocation of treatment combinations. A second order polynomial regression model was fitted and was found adequate, with an R^2 of 0.94469 ($P < 0.001$). The effects of temperature and pH were the most significant factors in influencing enzyme production. Estimated optimum conditions were as follows: pH 5.0, temperature, 32 °C and an incubation period of 91 h. Pectinesterase (PE), pectin lyase (PL), and cellulase activities were not detected. Pectinase production was partially constitutive. Pectin was degraded by the isolated strain of *K. wickerhamii* in the current study, and the pectinolytic activity is referred to as polygalacturonase (PG) activity. Crude enzyme extract was thermostable at various temperatures and, stimulated by the presence of Ca^{2+} ions but inhibited by other ions like Mg^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+} and Na^{+}.

Keywords: *Kluyveromyces wickerhamii*; Pectinase; Polygalacturonase; Response surface methodology

1. Introduction

Microbial pectinolytic enzymes are known to play a commercially important role in a number of industrial processes. Among these are retting of flax and other vegetable fibres, increasing the yield of fruit juice extraction, clarification and depectinisation of fruit juices, extraction of oils from vegetables and citrus peels, maceration of fruits and vegetables to give unicellular foods (Kilara, 1982), and reducing viscosity in concentrates (Fogarty and Kelly, 1983). Use of pectinolytic enzymes is preferred over mechanical methods for peeling of fruits and is widely being adopted for several fruits (Ben-Shalon et al., 1984; Donaghy and McKay, 1994; Pretel et al., 1997).

The economics of pectinase production may be enhanced by the selection of more productive mutants or genetically modified organisms (Blanco et al., 1997, 1998), which are not subject to catabolite

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repression or synthesise large quantities of the enzyme without the necessity of an inducer (Fogarty and Kelly, 1983). The commercial pectinases used in food industry often come from moulds, in particular the species of the genus *Aspergillus* (for example, *Aspergillus niger*, *A. wentii*, and *A. oryzae*) and *Rhizopus* (Acuña-Argüelles et al., 1995). Mould pectic enzymes normally contain a mixture of pectinolytic enzymes and are always associated with xylanases, cellulases and hemicellulases. However, there are cases where only one type of pectinolytic enzyme is required. The cloud in orange juice can be stabilised by use of high levels of polygalacturonase (PG). Many commercial pectinases form *A. niger* are low in PG activity. *A. niger* also secretes pectinmethylesterase which, if left in the pectinoltic digest, produces the toxic alcohol methanol. The production costs of PG from *A niger* are high if pure enzyme is needed since the separation from other enzymes is required. *A. niger* also secretes a variety of other enzymes. Some possess oxidising activities that are not desirable in the production of wines and fruit juices. Although they sometimes produced one or two types of pectinolytic enzymes, the production of pectinase is not so widespread in yeasts. Very few yeasts show this ability namely those belonging to the genera *Saccharomyces, Kluyveromyces, Cryptococcus, Rhodotorola,* and *Candida* (Luh and Phaf, 1954; Vaugn et al., 1969; Winborne and Richard, 1978; Lim et al., 1980; Federici, 1985; Barnby et al., 1990). Pectinases are not produced commercially from bacteria, although highly productive constitutive strains are known (Lei et al., 1985; Chatterjee et al., 1995). This is because of the high costs of production.

The conventional method that has been used for optimisation is the ''change-one-factor-at-a-time'' method in which a single factor or one independent variable is varied while fixing all others at a specific level may. This may lead to unreliable results and less accurate conclusions (Oh et al., 1995). Response surface methodology (RSM), which includes factorial designs and regression analysis, can better deal with multifactor experiments (Quintavalla and Paralari, 1993; Kalathenos et al., 1995; Nketsia-Tabiri and Sefa-Dedeh, 1995; Montgomery, 1997; Sen and Swaminathan, 1997). This present investigation has been carried out with an aim to isolate a pectinase-producing microorganism and to optimise culture conditions for the production of extracellular pectinolytic activity.

2. Materials and methods

2.1. Isolation and screening

Pectinase-producing organisms were isolated from various sources, with special attention given to rotting fruits such as apples, oranges, tangerines and pears obtained from the local shops. The pectinolytic microorganisms were selected on the basis of utilisation of pectin as a sole carbon source in the selective medium which contained (g/1000 ml distilled water): $CaCl_2$ (Saarchem 5822320, RSA), 0.05; KH_2PO_4 (Saarchem 5043610 RSA), 0.2; $MgSO_4.7H_2O$ (Saarchem 4123920, RSA), 0.8; $(NH_4)SO_4$ (Saarchem 1122720), 1.0; Yeast extract (Oxoid E21, England), 1.0; High Quality Agar (Oxoid LP0011), 15 and Citrus pectin (Sigma P9135, St Louis, MO), 0.05. Wherever possible, the fungal spores were taken directly from the infected material onto the selective medium. Alternatively, the plant material was blended and serially diluted in distilled sterile water before plating on the selective medium. The plates were incubated at 25 °C for 48 h. The colonies that were surrounded by a largest diameter of the transparent zone were selected as potential producers of pectinase. By sub-culturing through several generations in pectin agar, the stability in pectinolytic activity was ascertained.

2.2. Maintenance of cultures

Isolated yeast strains were maintained at 4 °C on agar slopes containing (g/l): yeast extract 3.0 g (Oxoid E21); peptone (Oxoid L49) 5.0 g; glucose Merck 108342 RSA), 20 g; malt extract (Oxoid CM57), 3.0 g and High Quality Agar, 15 (Oxoid LP0011), 20 g (Schawan and Rose, 1994).

2.3. Inoculum preparation

Forty-eight (48)-h cultures of yeast strains grown on malt extract agar (Oxoid CM59) plates at 25 °C were used to prepare standard inocula. Sterile distilled water (5 ml) was used to suspend the yeast cells. The suspension was adjusted to an optical density of 1.0 at

540 nm on a 21-UVD-spectronic spectrophotometer using a 1-cm light path test tube. Only 1 ml of the suspension per 100 ml of medium was used as a standard inoculum (unless otherwise stated), in 250 ml capacity Erlenmeyer flasks.

2.4. Pectinase production

Isolated pectinase-producing yeasts were grown in a modified media of Kebede (1994), which contained the following (g/l): NaCl (Saarchem 5822320, RSA), 0.1; $CaCl_2$ (Saarchem 152910, RSA), 0.02; KH_2PO_4 (Saarchem 5043610, RSA), 0.2; $FeCl_3.6H_2O$(-Saarchem 2340530, RSA), 0.001; NH_4Cl (Saarchem 1122720, RSA), 0.2; $MgSO_4$ (Saarchem 4123920, RSA), 0.17; Yeast extract, 0.5 (Oxoid E21), which was supplemented with a chosen carbon source. Citrus pectin (Sigma P9135, Germany), galacturonic acid (Sigma G2125, Germany), polygalacturonic acid (Sigma P3889, Germany), glucose (Merck 108342, Germany) glycerol (Merck 104092, RSA), cellobiose (Sigma C7252, Germany), xylose (Sigma X3877, Germany) and sucrose (Merck 10012085, RSA) were all used as carbon sources. All reagents were analytical grade. The carbon source and yeast extract were sterilised separately from the basal medium. The basal media were sterilised at 15 psi, 121 °C for 15 min. The total extracellular pectinase was determined in the culture supernatant after growth in an incubator shaker (Gallenkamp, UK) at 150 rev/min maintained at 30 °C with different combinations of temperature (24–48 °C), pH (3.0–5.0) and time of incubation (40–120 h). The initial pH was adjusted using 0.5 M HCL.

2.5. Preparation of enzyme samples

Samples from the medium were withdrawn periodically, centrifuged at 4 °C using Biofuge stratos (Heraeaus Intsruments, Germany) at 10,000 × *g* for 10 min. The cell-free extract was used to serve as a crude enzyme source of total pectinase, polygalacturonase, pectinesterase, pectin lyase and cellulase.

2.6. Assay for pectinase activity

Extracellular pectinase activity was measured by quantifying, using the method of DNS (Miller, 1959), the number of reducing groups which had been liberated after incubation with 1% citrus pectin solution, using galacturonic acid as a standard units. The assay was carried out using 1 ml of 1% pectin (dissolved in 0.05 M acetate buffer), 8.5 ml of acetate buffer (pH 5.0) and 0.5 ml of culture filtrate. The resulting mixture was incubated at 45 °C, with shaking for 1 h in a water bath incubator shaker. One unit of pectinase activity was defined as the amount of the enzyme, which catalysed the formation of 1 μmol of galacturonic acid per hour at 45 °C. The enzyme activity was expressed as units per milligram dry weight per millilitre (U/mg DW/ml) of the supernatant (Baracat et al., 1989).

2.7. Assay for polygalacturonase (PG)

Polygalacturonase activity was assayed by incubating a mixture of 1 ml of 1% polygalacturonic acid (dissolved in 0.05 M acetate buffer, pH 5.0), 8.5 ml of sodium acetate buffer and 0.5 ml of culture filtrate at 45 °C for 1 h in a water bath incubator shaker (Baracat et al., 1989). Polygalacturonase activity was measured by quantifying the amount of reducing sugar groups which had been liberated after incubation with 1% polygalacturonic acid at 45 °C, using the method of DNS (Miller, 1959) and using galacturonic acid as a standard. One unit of polygalacturonase activity was defined as the amount of the enzyme, which catalysed the formation of 1 μmol of galacturonic acid per hour at 45 °C. The enzyme activity was expressed as units microgram dry weight per millilitre (U/mg DW/ml).

2.8. Assay for pectinesterase activity (PE)

A 1% (w/v) solution of pectin (47% esterified) in 0.1 M sodium chloride was adjusted to pH 7.5 with 0.5 M sodium hydroxide. Unbuffered enzyme solution 0.1–5.0 ml was added to 20 ml of pectin solution, and the pH was maintained at 7.5 for 30 min by the addition of 0.02 M sodium hydroxide. The volume of sodium hydroxide added is proportional to enzyme activity (Barnby et al., 1990).

2.9. Assay for pectin lyase activity (PL)

Pectin lyase activity was measured spectrophotometically. One-half millimetre enzyme solution was

added to 0.25% (w/v) pectin in 0.1M Tris–HCL buffer, pH 7.5, and the increase in absorbance at 240 nm was monitored (Barnby et al., 1990).

2.10. Assay for cellulase activity (cellulase)

Cellulase activity was determined by measuring the release of reducing groups using DNS assay and glucose as a standard. The reaction mixture consisted of one strip of filter paper (1 × 7 cm) and 3.0 ml of culture supernatant was incubated at 45 °C for 1 h. One unit of cellulase activity (Cellulase unit) was defined as the micromoles of glucose released per microgram of protein per hour.

2.11. Reducing sugar determination

In all cases, the amount of reducing sugar was determined using 1 ml 3,5-Dinitrosalicyclic acid (DNS) reagent (Sigma D0550, Germany) and 1 ml of reaction mixture in a test tube (2 × 17 cm) and was boiled for 15 min. A 1 ml solution of 40% Potassium Sodium Tartarate (Rochelle salt) (Sigma P0165, Germany) was added to the mixture of the reactants subsequent to development of colour and prior to cooling. The reagent control contained for each assay contained 1 ml of substrate solution that had been incubated in the absence of the inoculum. After cooling, the optical density of resulting coloured mixture was read at 575 nm using 1-cm light path cuvette. D-Galacturonic acid was used as a standard with which the amounts of reducing sugars produced by the action of pectinases and polygalacturonases (Miller, 1959).

2.12. Biomass quantification

Protein quantification was carried out by the modified method of Lowry et al. (1951) using bovine serum albumin (BSA) (Sigma B2771, Germany) as a standard. Biomass determination of yeasts was determined gravimetrically after drying cells at 80 °C.

2.13. Experimental design

A central composite design for $k=3$ was used and it generated 20 treatment combinations (Cochran and Cox, 1959; Montgomery, 1997). This generated 20 sample combinations. The effects of independent variables, pH of growth medium, temperature, and fermentation time on the response (i.e. the pectinase production by the isolate *Kluyveromyces wickerhamii*) were investigated. Five levels of each variable were chosen. The upper and the lower limits of each variable were chosen to encompass the range in literature and to reflect what is done in practice after a preliminary investigation of the limits. The remaining levels were identified using CCRD (Montgomery, 1997). To set up a statistical model, we let Y denote Units of pectinase production, we determined code factor levels as follows: X_1=(temperature—35)/5.0, X_2=(pH—4.0)/0.5 and X_3=(time—72)/24. Table 1 Contains the actual factor levels corresponding to the coded factor levels. For each factor, a conventional level was set to zero as a coded level. Using this design, a second order polynomial regression model to the data (Eq. (1)) and was used to generate response surfaces. The treatment combinations and response are shown in Table 2.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + \varepsilon \quad (1)$$

2.14. Statistical analysis

Data were analysed using SPSS for Windows Release 6.1.3 (SPSS, USA) to yield regression equations, regression coefficients and analysis of variance.

Table 1
Process variables used in the central composite rotatable design ($K=3$) with actual factor levels corresponding to coded factor levels

Factor	Code[a]	Actual factor level at coded factor levels of:				
		−1.682[b]	−1	0	1	1.682
Temperature (°C)	X_1	26.59	30	35	40	43.4
pH	X_2	3.159	3.5	4.0	4.5	4.841
Time (h)	X_3	31.68	48	72	96	112.32

[a] Code level limits based on preliminary investigations and also to reflect what is done in practice. (X_1=(temperature−35)/5.0, X_2=(pH−4.0)/0.5 and X_3=(time−72)/24).
[b] Levels based on the Central Composite Rotatable Design (Cochran and Cox, 1959; Montgomery, 1997).

Table 2
Treatment combinations and mean responses

Treatment	Coded variable level[a]			Mean response (Y) (U/mgDW)
	X_1[b]	X_2	X_3	
1	−1	1	1	6.0
2	−1	1	1	7.0
3	1	−1	1	7.6
4	1	1	−1	4.5
5	0	0	0	8.8
6	0	0	0	8.1
7	−1	−1	1	3.5
8	−1	1	−1	7.5
9	1	−1	−1	7.9
10	1	1	1	7.8
11	0	0	0	7.8
12	0	0	0	7.7
13	1.682	0	0	7.0
14	−1.682	0	0	7.8
15	0	−1.682	0	6.7
16	0	−1.682	0	4.3
17	0	0	1.682	3.5
18	0	0	−1.682	2.2
19	0	0	0	2.7
20	0	0	0	1.5

[a] Code level limits based on preliminary investigations and also to reflect what is done in practice. (X_1=(temperature−35)/5.0, X_2=(pH−4.0)/0.5 and X_3=(time−72)/24).

[b] Levels based on the Central Composite Rotatable Design (Cochran and Cox, 1959; Montgomery, 1997).

The models were examined for lack of fit and adequacy and efficiency in predicting the response. All three-dimensional response surface graphs and two-dimensional contour plots were generated using STATISTICA for Windows (Release 5.1, Stasoft, USA). In our regression model, the response variable pectinolytic activity and candidates for explanatory variables are linear, interaction and quadratic terms of coded levels of temperature, pH and time. The α-level at which every term in the selected model should be significant was set at 5%. Optimum conditions were found through differentiation.

2.15. Effects of metal ions on enzyme activity

The effect Mg^{2+} ($MgCl_2$; Merck 814733 RSA), Ca^{2+} ($CaCl_2$; Merck 102383 RSA), Zn^{2+} ($ZnCl_2$; Merck 108813 RSA), Co^{2+} ($CoCl_2$; Merck 159242 RSA), and Mn^{2+} ($MnCl_2$; Merck 105917 RSA) on enzyme activity was investigated from 0–5 mM concentrations.

2.16. Thermal stability studies

Enzyme stability studies were performed by first incubating the culture filtrates in the absence of substrate at 50, 60 and 70 °C. Samples were withdrawn at various intervals up to 90 min and the residual pectinolytic activities were then determined. Incubating the culture filtrates in the presence of the metal ions and samples withdrawn periodically for enzyme assays, the effect of metal ions that enhance activity on thermal stability was assessed.

3. Results and discussion

Eighteen strains of fungi were isolated from rotting fruits based on growth and diameter of transparent zone around their colonies. Only five of these (four moulds, one yeast) had a significantly higher (>14 mm) diameter around their colonies. The moulds were identified as *Rhizopus* sp., *A. niger*, *Aspergillus* sp., and *Penicillium* sp. by morphological examination using light microscopy. Substantial work has been done on the moulds isolated in this study as regards pectinolytic activity. The yeast isolate identified as *K. wickerhamii* using the method of De Vroey and Raes-Wutyluck (1988) was found excrete reasonable stable extracellular pectinolytic activity on pectin agar gels and was used in this study. Some members of this genus have also been shown to be potential sources of the pectinase, especially *K. marxianus* of which 85–90% of the proteins it secretes are polygalacturonases (Barnby et al., 1990; Schwan et al., 1997).

The analysis of variance (ANOVA) demonstrates that the model is highly significant ($P<0.001$; Table 3) and the R^2 value being the measure of the goodness of fit of the model, indicates that 94.469% of the total

Table 3
Analysis of variance for the evaluation of the second-order model for the model

Source of variation	df	Sum of squares	Mean square	F-ratio	P-value
Model	9	205.19447	22.79939	56.92949	0.0000
Residual	30	12.01454	0.40048		
Total	39	217.20901			

R-square = 0.94469; Standard error of the estimate = 0.63284; adjusted R-square = 0.92809; Durbin–Watson test = 1.47109.

variation is explained by the model. In studying the influence of growth conditions on pectinase production, the model clearly reveals no significant interactions between temperature and time ($P>0.05$) and nonsignificant interactions between temperature and pH ($P<0.05$). Further, ANOVA (Tables 4 and 5) for variables in the model showed that the linear effect of temperature was the most important variable, as it contributed 39.206% of the variation of 94.469%. The quadratic effect of time also contributed significantly ($P<0.05$) to the model, as it accounted for 28.079% of the total variation. The linear effects of pH also contributed significantly ($P<0.05$) to the total variation. Thus, in the growth and enzyme production of *K. wickerhammi*, the fermentation time, initial pH of medium and temperature are all important and treating them separately may not reflect their true influence to the total variation or to the response. This model therefore indicates any of them and or a combination can be manipulated to control (enhance) growth and enzyme production.

The shapes of contour plots indicate the nature and extent of the interactions. Prominent interactions are shown by the elliptical nature of the contour plots (Fig. 1), while less prominent or negligible interactions would otherwise be shown by the circular nature of the contour plots. The more linear the response (Fig. 2), the less the magnitude of interactions and vice versa (Figs. 1, 3, 4 and 5). The response surface plot (Fig. 3) indicates an optimum enzyme activity around a pH of 3.8–4.5 and a temperature of 28.5–35.5 between 72 and 120 h of incubation.

Table 4
Coefficient estimates in the regression model selected through variable selection

Independent variable (parameter[a])	Coefficient (β)	Standard error, S.E. (β)	t-Value	Significant level
X_1*	−1.765645	0.121082	−14.582	0.0000
X_2*	−0.369988	0.121082	−3.056	0.0047
X_3*	0.917176	0.121082	7.575	0.0000
X_1X_1*	−1.545911	0.117855	−13.117	0.0000
X_1X_2	−0.025919	0.158210	−0.164	0.8710
X_1X_3**	−0.456090	0.158210	−2.883	0.0072
X_2X_2	−0.2283380	0.117855	−1.937	0.0622
X_2X_3*	0.472220	0.158210	2.985	0.0056
X_3X_3*	−0.928935	0.117855	−7.882	0.0000

[a] X_1, Incubation temperature (°C); X_2, pH; X_3, fermentation time.
* Highly significant ($P<0.01$).
** Significant ($P \leq 0.05$).

Table 5
Further ANOVA for the variables in the order fitted

Source	*df*	Mean square	*F*-ratio	*P*-value
Temperature	1	85.15926	24.50631	0.0000
pH	1	3.73938	0.66565	0.4197
Time	1	22.97894	4.49570	0.0406
Temperature^2	1	60.99063	14.83592	0.0004
Time^2	1	17.34985	3.29693	0.0773
pH^2	1	0.00061	0.00011	0.9918
pH*time	1	3.56787	0.63461	0.4306
Temperature*time	1	3.32829	0.59133	0.4467
Total	7	193.37545		

The seven-variable model was identified using stepwise multiple regression analysis. The functional form of the model (Eq. (1)) was solved by partial differentiation. The maximum values of the process variables in coded values are as follows: $X_1=-0.6968818$; $X_2=0.3919087$; and $X_3=0.7835076$ with corresponding $\acute{Y}=8.510477$ U/mg DW. The uncoded values of the test variables are as follows: temperature = 31.52 °C, pH = 4.70 and time = 90.80 h. The experimental results indicated a maximum yield of pectinolytic activity of 7.9475 U/mg DW, which confirms the closeness of the model to the experimental results.

The enzyme activity of 8.51 U/mg DW/ml is close to the optimum pectinolytic activity of *Aspergillus sp.* CH-Y-1043 of 10 U/mg DW/ml (Aguillar et al., 1991). The optimum pH and temperature optimum are also consistent with values that have been found for fungal pectinase production of 4–6 and 26–37 °C, respectively (Luh and Phaf, 1954; Lim et al., 1980; Whitaker, 1984; Schwan et al., 1997).

The commercially available preparations of pectinolytic enzymes are produced from *Aspergillus* sp. They contain a mixture of enzymes and are sometimes associated with cellulases and have usually low PG activity (Aguillar and Huitron, 1986; Acuña-Argüelles et al., 1995; Madden, 1995). Such mixtures are usually useful for extraction of juice and maceration of vegetables. On the other hand, only one type of pectinolytic activity may be required, for instance, in the stabilisation of the cloud in orange juice, high levels of polygalacturonase are required (Kotzekidou, 1991). The organism in this study appears to be useful

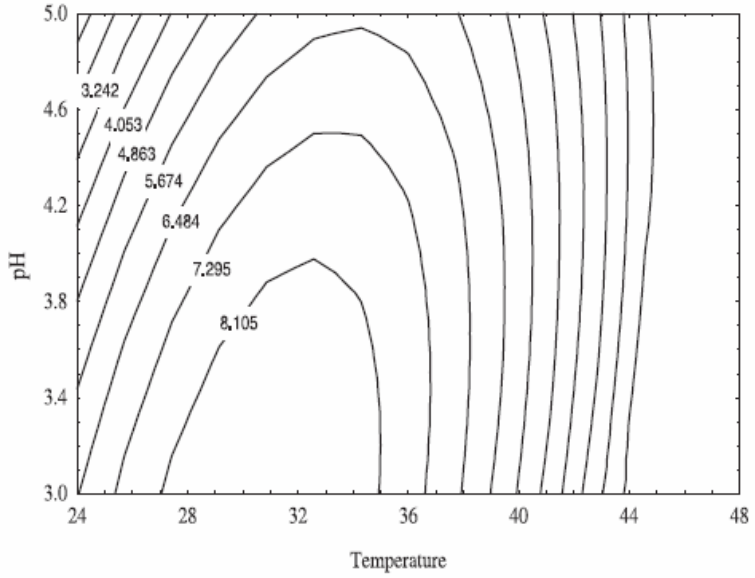

Fig. 1. Contour plots for the effect pH and temperature on the pectinolytic activity of *K. wickerhammi* after 72 h of incubation. Figures shown indicate the level of pectinolytic activity in Units per milligram dry weight per ml (U/mg DW/ml). One unit (U) of pectinase activity was defined as the amount of the enzyme, which catalysed the formation of 1 μmol of galacturonic acid per hour at 45 °C.

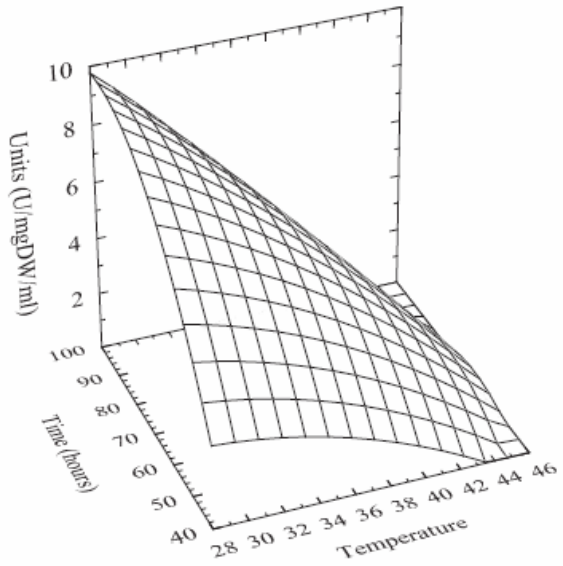

Fig. 2. Response surface for the effects of temperature (°C) and time (h) at pH 4.5 on the pectinolytic activity of *K. wickerhamii*. One unit (U) of pectinase activity was defined as the amount of the enzyme, which catalysed the formation of 1 μmol of galacturonic acid per hour at 45 °C.

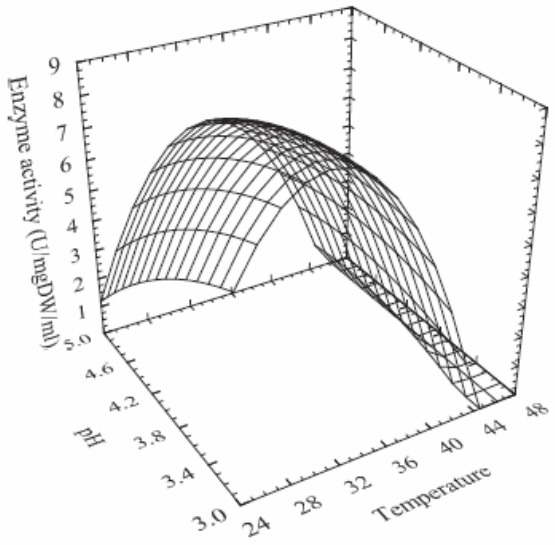

Fig. 3. Response surface for the effects of pH and temperature (°C) on the pectinolytic activity of *K. wickerhammi* after 72 h of incubation. One unit (U) of pectinase activity was defined as the amount of the enzyme, which catalysed the formation of 1 μmol of galacturonic acid per hour at 45 °C).

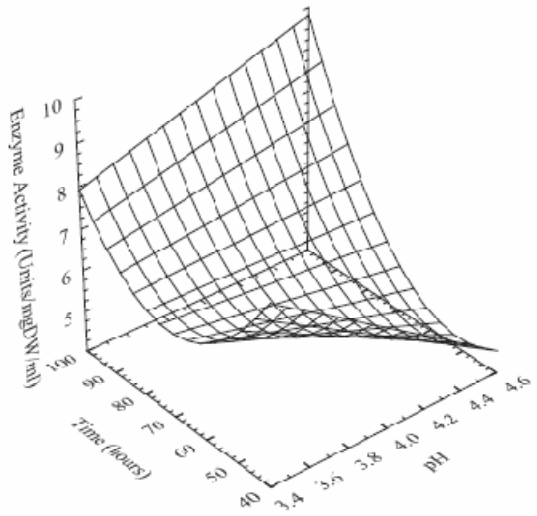

Fig. 4. Response surface for the effects of pH and time on the pectinolytic activity of *K. wickerhammi* at 30 °C. One unit (U) of pectinase activity was defined as the amount of the enzyme, which catalysed the formation of 1 μmol of galacturonic acid per hour at 45 °C.

for such purposes and is a potential source of a pure PG enzyme, which could be used directly in fruit and vegetable processing or on cocoa beans for the extraction of cocoa juice. Since *K. wickerhamii* produced one type of pectinolytic activity (PG activity), it can be used as a direct source of the enzyme, PG. This may reduce the time and cost involved in the production of PG. This is because the current source *A. niger* produces as wide range of pectinases and other enzymes. The separation of PG from other enzymes is very expensive.

Pectinase production has been shown to be influenced by the type and source of carbon (Nair et al., 1995). When the isolate was grown on glucose, as a sole carbon source pectinase, levels remained lower than when pectin was used as a sole carbon source. Pectinase was produced in the absence of pectin (Fig. 6) suggesting the constitutive nature of the extracellular pectinase. It has been demonstrated that bacteria such as *Bacillus subtilis* (Fogarty and Kelly, 1983), moulds such as *Aspergillus* sp. CH-Y-143 (Aguillar and Huitron, 1990), are capable of producing polygalacturonases constitutively. The absence of growth observed with 1% galacturonic acid as a sole carbon source may suggest some form of catabolite repression (Tahara et al., 1975; Solis-Perera et al., 1993; Schawan and Rose, 1994).

An increase in extracellular pectinase activity in the combination of glucose (1%) and pectin (1%), as compared to the individual substrates, suggests that the extracellular pectinase production of the *K. wickerhammi* was partially constitutive. The presence of pectin (1%) in the media containing glucose (1%) thus induced an increase in enzyme activity from 9.0 to 13.7 U/mg DW/ml, which represents an approximate 50% increase in the pectinolytic activity. Winborne and Richard (1978) have observed this effect in a similar study of the yeast *Saccharomyces fragilis* (*Kluyveromyces fragilis*). Since pectin is a high-level molecular weight polysaccharide, the question arises as to how induction takes place or how the cells can sense the substrate in the outer environment. It has been suggested that some microorganisms can produce low levels of basal constitutive activities that degrade the polymeric substance, and that low levels of the reaction serve as inducers or energy sources to

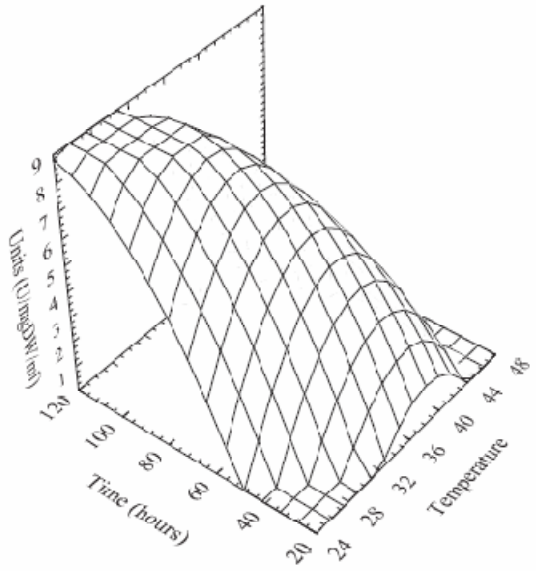

Fig. 5. Response surface for the effect of temperature with time at pH 4.0 on the pectinolytic activity of *K. wickerhammi*. One unit (U) of pectinase activity was defined as the amount of the enzyme, which catalysed the formation of 1 μmol of galacturonic acid per hour at 45 °C.

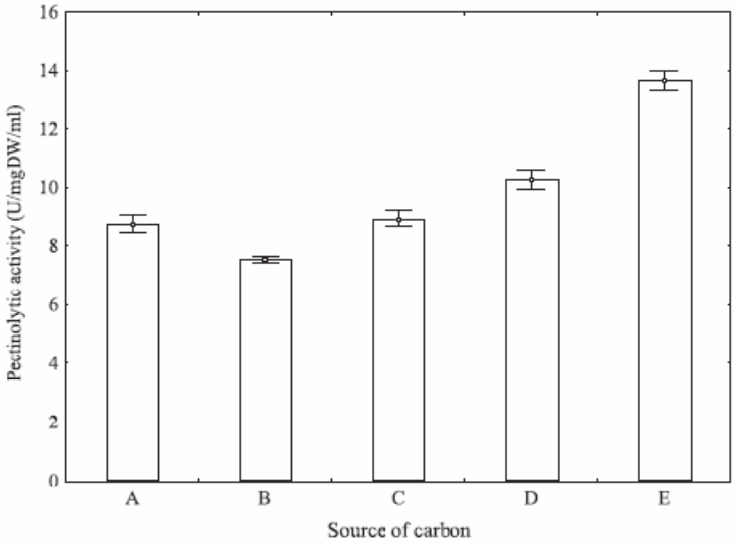

Fig. 6. Effect of carbon sources on the pectinolytic activity of *K. wickerhammi*. One unit (U) of pectinase activity was defined as the amount of the enzyme, which catalysed the formation of 1 μmol of galacturonic acid per hour at 45 °C. A=Pectin (1%), B=Glucose (0.2%), C=Pectin (1%)+glucose (0.2%), D=Glucose (1%), E=Pectin (1%)+glucose (1%).

promote cell growth and pectinase production (Leone and Van den Heuvel, 1986; Aguillar and Huitron, 1987). This has been demonstrated in some bacteria and fungi (Kelly and Forgarty, 1978; Dosanjh and Hoondal, 1996; Aguillar and Huitron, 1987; Schawan and Rose, 1994; Schwan et al., 1997).

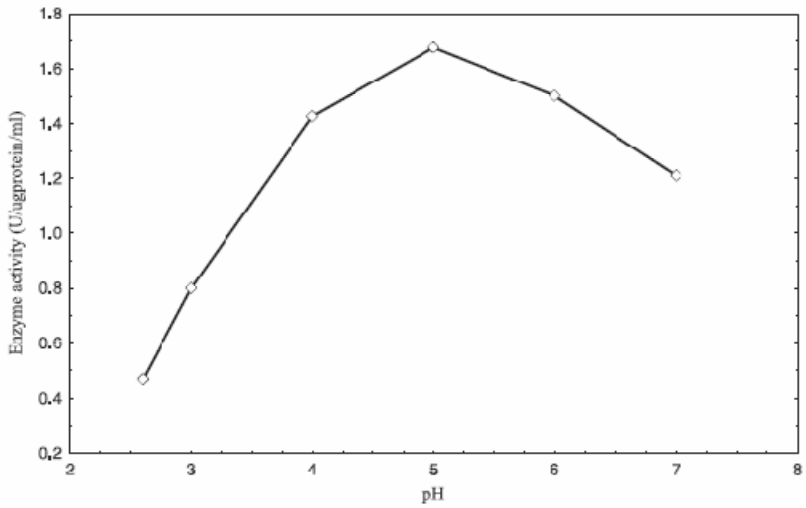

Fig. 7. The effect of pH on enzyme activity of the crude pectinase extract of *K. wickerhamii*. One unit (U) of pectinase activity was defined as the amount of the enzyme, which catalysed the formation of 1 μmol of galacturonic acid per hour at 45 °C. (Cell-free culture supernatant from a 96-h culture maintained at pH 5.0 at 30 °C was used.)

The pectinase secreted by *K. wickerhamii* has optimum pH and temperature of 5.0 and 50 °C (Figs. 7 and 8, respectively) typical of pectinase secreted by most yeasts (Luh and Phaf, 1954; Sanchez et al., 1984; Ravelomanana et al., 1986; Barnby et al., 1990). The effect of temperature on the pectinase from *K. wickerhamii* was similar to that reported for other yeast like *S. fragilis, Kluyveromyces marxianus*, and *Candida* sp. (Lim et al., 1980; Sanchez et al., 1984; Ravelomanana et al., 1986; Barnby et al., 1990). The presence of up to 3 mM Ca^{2+} (Calcium chloride) stimulated enzyme activity by up to 16.46% over native enzyme activity (Table 6). Mg^{2+}, Zn^{2+}, Co^{2+}, $Mn^{2+}$and Na^{+} inhibited the pectinolytic activity of *K. wickerhamii,* unlike the pectinase of *Bacillus* GK-8 that has been shown to be stimulated by these cations (Dosanjh and Hoondal, 1996). Ca^{2+} has been reported to cause 290% increase in polygalacturonase lyase activity (Kelly and Forgarty, 1978).

The extracellular pectinase of our strain of *K. wickerhamii* was relatively thermostable as it retained over 80% for activity for 45–50 min at 60 and 70 °C in the presence of 3 mM Ca^{2+} ions. This suggests that Ca^{2+} conferred stability to the pectinase (Fig. 9a,b, and c). This may be due to the protective action by calcium chloride against heat inactivation of the pectinase. Similar results have been obtained in the production of pectinase by *Bacillus pumilus* (Fogarty and Kelly, 1983).

Table 6
Effects of metal ions on the activity of the pectinase of *K. wickerhamii*

Metal ions	Concentration (mM)					
	0	1	2	3	4	5
NA[a]	1.616 U/μg prot/ml					
Ca^{2+}		7.0%[b]	6.6%	16.5%	6.3%	5.5%
Mg^{2+}		No activity detected				
Zn^{2+}		No activity detected				
Co^{2+}		No activity detected				
Mn^{2+}		No activity detected				
Na^{+}		No activity detected				

[a] NA=native enzyme activity. Values quoted as Units (U/μg prot/ml).
[b] Percentage over native enzyme activity.

The thermostability of the enzyme is important in juice extraction. Prior to the use of pectinases, fruits

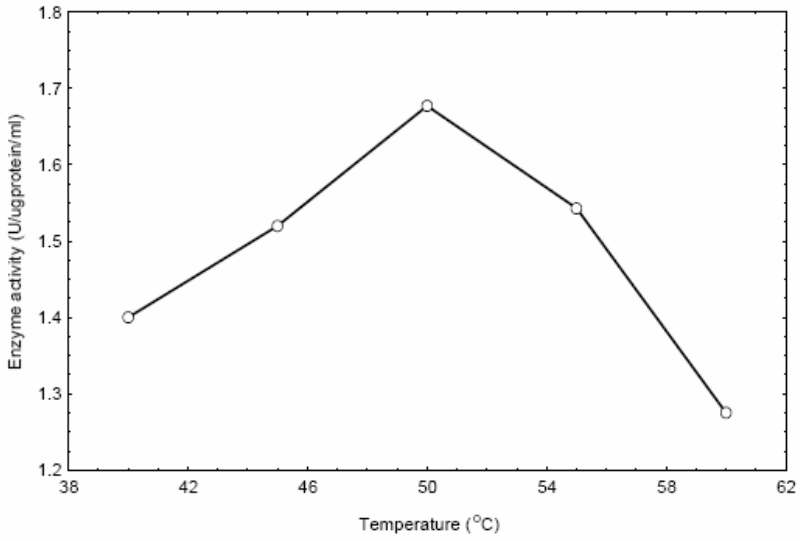

Fig. 8. The effect of temperature on the enzyme activity of the crude pectinase extract of *K. wickerhamii*. One unit (U) of pectinase activity was defined as the amount of the enzyme, which catalysed the formation of 1 μmol of galacturonic acid per hour at 45 °C. (Cell-free culture supernatant from a 96-h culture maintained at pH 5.0 at 30 °C was used.)

were first cooked to release more juice. This gave a cooked flavour and also released a lot of pectin into the juice giving a thick and a cloudy appearance. Apples, stone fruits and berries are normally processed at 30–50 °C for about 15 to 90 min (Madden, 1995). The fact that the pectinase from *K. wickerhamii*

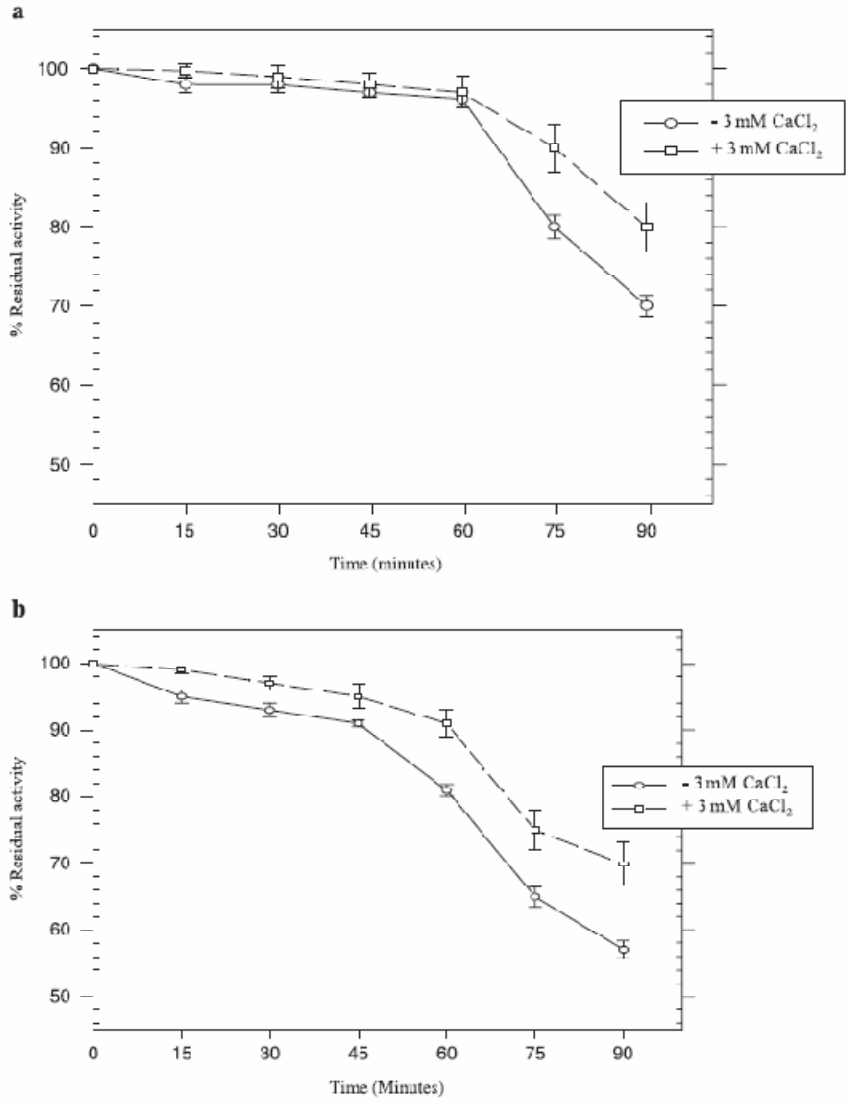

Fig. 9. (a) Thermal stability profiles for the pectinase of *K. wickerhamii* at 50 °C in the presence and absence of 3 mM $CaCl_2$. (Residual activity is the amount of enzyme activity that was retained over native (original) enzyme activity.) (b) Thermal stability profiles for the pectinase of *K. wickerhamii* at 60 °C in the presence and absence of 3 mM $CaCl_2$. (Residual activity is the amount of enzyme activity that was retained over native (original) enzyme activity.) (c) Thermal stability profiles for the pectinase of *K. wickerhamii* at 70 °C, in the presence and absence of 3 mM $CaCl_2$. (Residual activity is the amount of enzyme activity that was retained over native (original) enzyme activity.)

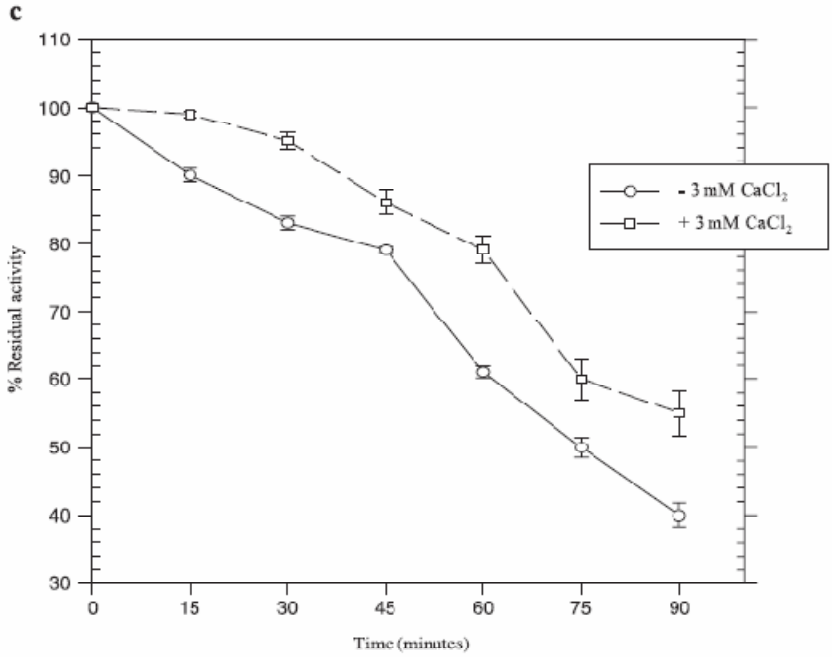

Fig. 9 (*continued*).

is thermostable up to 105 min at 50 °C and has an optimum temperature 50 °C presents excellent opportunities for its use in the fruit juice industry.

4. Conclusion

The conventional approach to optimisation used for multifactor experimental design is the "change-one-factor-at-a-time" traditionally used by many microbiologists has limitations because: (a) it generates large quantities of data which are often difficult to interpret, (b) it is time consuming and expensive and (c) ignores the effects of interactions among factors, which have a great bearing on the response.

The yeast strain of *K. wickerhamii*, which has been isolated from rotting fruits, is a potential source of pectinase for use in food industry if it is further explored. A more long-term goal would be to construct overproducing strains, which could be used as source of polygalacturonase and a starter cultures for fermentation. The optimum pectinolytic activity was achieved at pH 5, 33 °C after 91 h of incubation. The pectinase production was partially constitutive and the secreted pectinase was thermostable and its activity was enhanced by addition of 3 mM Ca^{2+} ions.

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