

Research Article

Kinetic Activity of Commercial Native Linamarase (CNLIN) and Engineered (β -glucosidase) from *Saccharomyces cerevisiae* on Cassava Linamarin

¹Julius Kwagh-Har Ikya, ¹Charles Chukwuma Ariahu and ²James Ortese Ayatse

¹Department of Food Science and Technology, University of Agriculture,
Makurdi, Benue State, Nigeria

²Federal University Dutsin-Ma, Katsina State, Nigeria

Abstract: The ability of Commercial Native Linamarase (CNLIN) and Engineered Linamarase (GELIN) extracts from *Saccharomyces cerevisiae* to hydrolyse cassava linamarin was challenged. CNLIN acting as control was used together with GELIN extracts from *Saccharomyces cerevisiae* to evaluate the kinetic data for test enzymes at pH 3.5, 6.8 and 10.5, respectively and ambient temperature (35°C). Data obtained from the varying activity versus substrate concentrations were fitted with the Michaelis-Menten plots and Lineweaver-Burk model to obtain maximum velocity (V_{max}), affinity coefficient (K_m), physiological efficiency (K_m/V_{max}) and r^2 (linear regressing coefficient). The results indicated that the engineered linamarase conferred different enzyme kinetic data showing degradation of cassava linamarin by CNLIN and GELIN from *Saccharomyces cerevisiae* at the optimum pH and temperature. The relation was best described by the characteristic sigmoid Michaelis-Menten plots and Lineweaver-Burk model evidence from the high coefficient of linear regression ($r^2 > 0.976$). V_{max} and K_m derived from the Lineweaver-Burk model varied from 10.0 to 13.0 $\mu\text{mol}/\text{min}$ and 0.5 to 0.9 μM respectively for engineered enzymes and 0.0-10.0 mol/min and 0.0 to 0.9 μM respectively for CNLIN. The kinetics profiles of the studied enzymes showed their actions on cassava linamarin were influenced by degree of genetic manipulation, purification and pH at ambient temperature. The wide pH tolerance in the degradation of linamarin suggests a possible use of the engineered linamarase from *Saccharomyces cerevisiae* in detoxifying linamarin in cassava for the production of cyanide-free cassava-based food products.

Keywords: Enzyme kinetics, linamarin, lineweaver-burk, michaelis-menten, pH, *Saccharomyces cerevisiae*

INTRODUCTION

Background information: Linamarase (β -glucosidase) is a hydrolytic enzyme. It degrades the glycolytic bond between β -glucose molecule and the chiral carbon atom linked to the nitrile group of linamarin present in cassava. Linamarase (β -glucosidases) is economically very important because of its role as a detoxification agent for improving food safety. Even though it can be produced from cassava (*Manihotesculenta crantz*) tubers the yield of native linamarase from this source is usually too low and expensive such that it is economically unwise to produce the enzyme from cassava tubers (Nok and Ikediobi, 1999). The problems associated with native linamarase (β -glucosidases) (CNLIN) used for detoxification include low enzyme concentration, limited spectrum of substrates activity and sensitivity to environmental factors such as pH and temperature. Over the years however, humans have realized that selective propagation of animals and plants of desirable traits can improve and increase enzyme production in yield and quality. β -glucosidases of

microbial origin may represent a possible solution to the problem. This approach has also been extended to bacteria and fungi for the increased downstream products development for the pharmaceutical, food and brewing industries. Yeast (*Saccharomyces cerevisiae*) for example, has long been used for the production of high yielding metabolites and other food enzymes including amylases and zymase for hydrolysis of starch and fermentation of glucose into ethanol respectively (Mach and Zeilinger, 2009). Studies by Ikediobi and Ogundu (1985) and Okafor and Ejiofor (1990) have showed that some quantities of linamarase can be extracted from yeast. Yeast like other microorganisms has been extensively used in genetic engineering for the production of single-cell proteins, enzymes, hormones and vitamins. This is because of their low generation times and ease of manipulation. The kinetic data of engineered β -glucosidase on linamarin extracted from cassava will provide insight into the mechanism of action of the enzyme while providing the parameters necessary for predictive purposes. Such predictions which are lacking can be useful tools for fermentation

Corresponding Author: Julius Kwagh-Harikya, Department of Food Science and Technology, University of Agriculture, P.M.B. 2373, Makurdi, Benue State, Nigeria, Tel.: +234 0816 067 6702

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process optimization for the degradation of cyanogenicglucosides in cassava-based food systems.

Research objective: The research objective of this study was to evaluate the kinetic data such as maximum velocity (V_{max}), affinity coefficient (K_m) and physiological efficiency (K_m/V_{max}) and r^2 (linear regressing coefficient) for the test enzymes at pH 3.5, 6.8 and 10.5 and ambient temperature (35°C). The approach is to boost products transformation and development of cassava-based food products that can contribute to global nutrition and food security, increase international export earnings from cassava for Africa countries.

MATERIALS AND METHODS

Materials:

Enzyme samples: Commercial Native Linamarase (CNLIN) used as the control for this study was purchased from Sigma Co. Lousina, USA. Engineered β -glucosidase (GELIN) was obtained from the Department of Food Science and Technology, University of Agriculture Makurdi. Crude extract from *Saccharomyces cerevisiae* (GELIN₀) was further purified using Carboxy methyl cellulose (GELIN₁), Diethyl-amino-ethyl-sephadex (GELIN₂) and Diethyl-amino-ethyl-cellulose (GELIN₃)

Substrate: Linamarin (100 g) was extracted from about 2kg tubers of the bitter wild cassava (*Manihotesculentapohr*) variety TSM-TRF-2005035 obtained from Tse-Akaa Village MbalaghMakurdi, Benue State, Nigeria and produced using the method described by Ikediobi and Ogundu (1985): One year old cassava tubers were harvested washed with tap water and promptly frozen overnight at -10°C. About 800 g of frozen cassava parenchyma tissues were sliced with stainless steel knives and homogenized with 160 mL of chilled 0.1 M phosphoric acid solution. The resultant slurries were filtered rapidly using glass wool and the filtrate centrifuged (1000 rpm) for 5 min. The resultant filtrate was centrifuged at 5000×g for 5 min and the supernatant adjusted to pH 8.0 followed by re-centrifugation at 5000×g for 8 min. After decanting, the solid residue was air-dried to obtain about 0.82 g of white substrate (mp.143°C). This was stored at 4°C for subsequent use for enzyme kinetics studies.

Buffer solutions and analytical grade reagents: Buffer solutions and reagents were prepared for the study using standard methods.

Kinetic studies: The ability of Commercial Native Linamarase (CNLIN) purchased as control and the Engineered Linamarase (GELIN) extracts from

Saccharomyces cerevisiae to hydrolyse cassava linamarin to Hydrocyanic acid (HCN) released within a fixed time was challenged. The spectrophotometric method was used for the estimation of Hydrocyanic acid (HCN) released within a fixed time as described by Onyike *et al.* (2001).

Procedure: Enzyme aliquots (0.1 mL) were prepared and added to tubes containing (1, 2, 3, 4, 5, 6, 7, 8 and 9, respectively) $\mu\text{mol}/\text{dm}^3$ each of linamarin. The tubes were incubated for 30 min at 30°C at varying pH 3.5, 6.8 and 10.5. The reaction was stopped by adding 1 mL of 0.2M NaOH followed by neutralization with 1 mL of 0.2 M HCl. Then 1 mL of 1% Chloramine T was added and followed after 1 min by addition of 3 mL barbituric acid/pyridine reagent. The volume was adjusted to 25 mL within and the absorbance of the pink color was measured at 420 nm. HCN was used to calibrate the absorbance values. The Hydrocyanic acid (HCN) present in each tube was determined spectrophotometric method. The absorbance measured in the enzyme assay was used to calculate the activity in units per mL of enzyme solution, where one unit is defined as that which produces 1micro mole of HCN per minute at 30°C. The total activity of the enzyme samples were calculated as described by Onyike *et al.* (2001):

Total activity = units which an enzyme required to totally hydrolyze 1 mg. of linamarin to Hydrocyanic acid (HCN).

Evaluation of michaelis-menten and line weaver-burk kinetics parameters: Data obtained from the varying activity versus substrate concentrations were fitted with the Michaelis-Menten plots to obtain sigmoid curves and linear regression analysis using Lineweaver-Burk model to obtain kinetic data such as maximum velocity (V_{max}), affinity coefficient (K_m) and physiological efficiency (K_m/V_{max}) for the test enzymes and r^2 (linear regression coefficient) at pH 3.5, 6.8 and 10.5 and ambient temperature (35°C), respectively.

The activity versus concentrations graphically presented as Michaelis-Menten kinetic plots at varying pH 3.5, 6.8 and 10.5 and ambient temperature (35°C) were used to confirm the enzymic behavior of the extracts while least square linear regression analysis of Line weaver-Burk kinetic model was applied in this study to validated the physiological characteristics of the enzymes.

Enzyme kinetic models: The initial rate of an enzyme catalyzed reaction can be related to substrate concentration using the Michaelis-Menten model as described by Ikediobi and Onyike (1987):

$$V_0 = \frac{V_{max} (S)}{K_m + (S)} \quad (1)$$

where,

- V_0 = Initial rate ($\mu\text{mol}/\text{min}$)
- V_{max} = Maximum velocity ($\mu\text{mol}/\text{min}$)
- K_m = (mol/dm^3)
- S = Substrate concentration (mol/dm^3)

Rearrangement of Eq. (2) gives:

$$k_m = (S) \left[\frac{V_{\text{max}}}{V_0} - 1 \right] \quad (2)$$

Linearization of Eq. (1) provides the Line weaver-Burk model as described by Nok and Ikediobi (1999) and shown in Eq. (3):

$$\frac{1}{v_0} = \frac{K_M}{V_{\text{Max}}} \frac{1}{(S)} + \frac{1}{V_{\text{max}}} \quad (3)$$

The value of the K_m reflects the stability of the enzyme-substrate interaction and is of great practical value. K_m is not however, the true dissociation constant of the enzyme-substrate complex (Nok and Ikediobi, 1999).

Statistical analysis: The tests for significant ($p \leq 0.05$) difference in the activity and kinetic parameters were calculated with the multiple comparison range method of Krammer and Twigg (1970). The least square linear regression analysis of the activity and kinetic parameters were calculated as described by Van-Boekel (1996) and Gupta (1979).

RESULTS AND DISCUSSION

Results:

Kinetic data: Plots of activity versus Linamarin concentration 1-9 mol/dm^3 for Commercial Native

Linamarase (CNLIN) and Engineered Linamarase groups (GELIN₀-GELIN₃) at pH 3.5, 6.8 and 10.5, respectively are shown in Fig. 1. The plots are typical of Michaelis-Menten model. Action of CNLIN on Linamarin was observed only at pH 6.8 whereas GELIN group acted on linamarin at all experimental pH (3.5-10.5) investigated. At pH 6.8 GELIN₃ gave the highest activity followed by GELIN₂, GELIN₁ and GELIN₀ in that order. The pattern was similar at pH 3.5 and 10.5. The regression parameters representing Lineweaver-Burk derivatives from Fig. 1 are showed in Table 1. The high coefficients of linear regression ($r^2 \geq 0.98$) indicated that Line weaver-Burk model was appropriate for describing the effect of linamarin concentration on the enzyme activity. The physiological efficiency K_m/V_{max} was between 0.5 to 1.25 min/dm^3 .

Discussion: Michaelis-Menten plots of activity versus Linamar in concentration 1-9 mol/dm^3 for Commercial Native Linamarase (CNLIN) and engineered linamarase groups (GELIN₀-GELIN₃) at pH 3.5, 6.8 and 10.5, respectively as shown in Fig. 1, produced sigmoid curves. Commercial Native Linamarase (CNLIN) acted on linamarin only at pH 6.8. The data derived from the enzyme activity versus substrate concentrations plots were used to estimate the physiological characteristics in accordance with those obtained by Onyike *et al.* (2001) and Nok and Ikediobi (1999). The total activity or maximum velocity (V_{max}) of (9-14 μmol HCN/min) fell within the range reported by earlier workers such as Onyike *et al.* (2001) for linamarin degradation by native β -glucosidase. Michaelis and Menten determined that the initial rate or velocity of catalysis of an enzyme varied hyperbolically with substrate concentration (Voet and Voet, 1995; Lionel *et al.*, 2008). In this present study, the initial velocity (V_0) generated in the degradation reactions were also subjected to Michaelis-Menten and Lineweaver-Burk

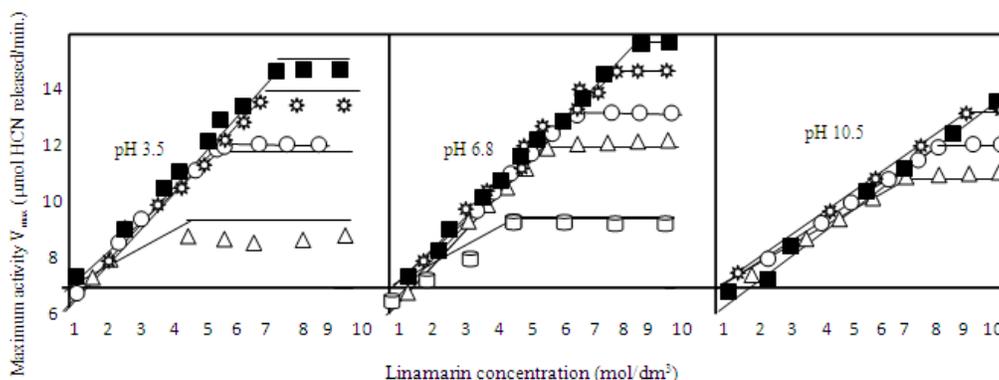


Fig. 1: Variation of maximum activity (V_{MAX}) of linamarase from *S. cerevisiae* with linamarin concentration
 ■: GELIN₃; ⚙: GELIN₂; ○: GELIN₁; △: GELIN₀; ◻: CELIN; CNLIN: Commercial native linamarase; GELIN₀: Crude Genetically Engineered Linamarase (GELIN); GELIN₁: 1st degree of purification using Carboxy-Methyl Cellulose (CMC); GELIN₂: 2nd degree of purification using Diethyl Amino Ethyl-sephadex (DEAE-sephadex); GELIN₃: 3rd degree of purification using Diethyl Amino Ethyl-cellulose (DEAE-cellulose)

Table 1: Lineweaver-burk parameters for degradation of linamarin by commercial and engineered linamarase from yeast (*Saccharomyces cerevisiae*)

pH	Lineweaver-burk parameters	Commercial and engineered linamarase				
		CNLIN	GELIN ₀	GELIN ₁	GELIN ₂	GELIN ₃
3.5	N	NAS	9	9	9	9
	R ²	NAS	0.980	0.980	0.980	0.980
	S.E.	NAS	0.320	0.310	0.290	0.250
	Intercept	NAS	0.250	0.135	0.080	0.071
	∴ V _{max} (μmol/min)	NAS	4	8	12	14
	K _m /V _{max}	NAS	1.250	0.750	0.580	0.570
	∴ K _m (mol/dm ³)	NAS	5	6	7	8
6.8	N	9	9	9	9	9
	R ²	0.98	0.980	0.980	0.980	0.980
	S.E.	0.31	0.260	0.230	0.190	0.150
	Intercept	0.25	0.135	0.100	0.071	0.063
	∴ V _{max} (μmol/min)	4	8	10	14	16
	K _m /V _{max}	1.25	0.625	0.600	0.200	0.200
	∴ K _m (mol/dm ³)	4	5	6	7	8
10.5	N	NAS	9	9	9	9
	R ²	NAS	0.980	0.980	0.980	0.980
	S.E.	NAS	0.130	0.120	0.120	0.120
	Intercept	NAS	0.170	0.135	0.100	0.083
	∴ V _{max} (μmol/min)	NAS	6	8	10	12
	K _m /V _{max}	NAS	0.830	0.750	0.700	0.670
	∴ K _m (mol/dm ³)	NAS	5	6	7	8

CNLIN: Commercial native linamarase Engineered Linamarase (GELIN); GELIN₀: Crude GELIN; GELIN₁: 1st degree of purification using Carboxy-Methyl Cellulose (CMC); GELIN₂: 2nd degree of purification using Diethyl Amino Ethyl-sephadex (DEAE-sephadex); GELIN₃: 3rd degree of purification using Diethyl Amino Ethyl-cellulose (DEAE-cellulose); NAS: No action on substrate; n: Number of points

models in line with earlier applications by Onyike *et al.* (2001) and Ikediobi and Ogundu (1985) for native linamarase extracted from cassava. The findings showed that the initial rates increased with increase in substrates concentration to a point where it reached maximum velocity (V_{max}). At low substrate concentrations, initial rate was proportional to the substrate concentration and can be referred to as first order kinetics. At high substrate concentrations, the initial rates were independent of substrates concentration and could be referred to as saturation or zero order kinetics. These results including sigmoid curves and the high coefficients of linear regression (r²≥0.98) values validated the adequacy of Michaelis-Menten and Line weaver-Burk models in describing the enzyme activity versus substrates concentration relationship.

The V_{max} (mol/min) at optimum pH of 6.8 were 10 (CNLIN), 10 (GELIN₀), 11 (GELIN₁), 12 (GELIN₂) and 13 (GELIN₃) respectively. The V_{max} values (mol/min) at optimum temperature of 35°C were not significantly (p<0.05) different. Those for linamarin degradation ranged from 4-14 (mol/min) at pH 3.5, 4-16 (mol/min) at pH 6.8 and 6-12 (mol/min) at pH 10.5 whereas K_m varied from 5 to 8 mol/dm³ at pH 3.5-10.5 for the engineered enzymes. Dounghkamol *et al.* (2006) reported K_m values of 1.02 to 5.04 mM at pH 6.0 and 0.01-0.05 mM at pH 3.5. The dissimilarity in K_m values could be due to differences in sequence identity. K_m values represent the degree of binding or affinity of the enzymes with substrates in formation of activated complexes necessary for formation of products. High

values indicate that higher attractive forces exist between substrates and enzymes with high probability of degradation of the cyanogenic glucosides. Chulavatnatol (2008), obtained K_m values ranging from 0.38 to 0.57 mM while Keresztzessy *et al.* (1994) reported K_m value of 1.06 mM for linamarin degradation and 0.36 mM for PNPg degradation. The higher values obtained in this study demonstrates the substrates specificity of engineered linamarase from *Saccharomyces cerevisiae* for the cyanogenic glucosides studied. The high apparent K_m values obtained for linamarin degradation indicated good affinity of the enzymes towards the substrate, particularly if compared to those of other β-glucosidases such as, for instance, the amygdalases of *Prunusserotina* (Kuroki and Poulton, 1986) and linamarase of *Penicillium aurantiogriseum* P35 (Petruccioli *et al.*, 1999b). For instance, β-glucosidases from *Brevibacterium* sp. and *A. oryzae* were active on linamarin at neutral pH (Legras *et al.*, 1989; Flores *et al.*, 1992); those from cassava and flax seeds were active on linamarin slightly above neutral pH (Yeoh, 2009), those from *P. serotina* and *Prunusolystachya* did not hydrolyzelinamarin at low pH while those from *P. aurantiogriseum* P35 acted on a wider spectrum of substrates at neutral pH (Petruccioli *et al.*, 1999a). The V_{max} values for the engineered linamarase (10.02-13.0 μmol/min) were higher than for CNLIN (0.0 to 10.0 μmol/min). The K_m value for the Commercial Native Linamarase (CNLIN) was 0.0 to 0.4 mol/dm³. This range is in agreement with that reported by Seshadi *et al.* (2010). As for physiological efficiency (K_m/V_{max}),

the crude engineered linamarase performed better than the purified enzymes at pH 3.5 with a value of 1.25 dm³/min. CNLIN had no action at this pH 3.5 and 10.5. At pH 6.8, the physiological efficiency of native linamarase was better than the engineered enzymes. At pH 10.5, the engineered enzymes degraded the cyanogenic glucosides and can therefore be applied exogenously for fermentation purposes to degrade these toxic food substances. These results are in agreement with the study of Petruccioli *et al.* (1999a) who's K_m/V_{max} was as high as 1.6 dm³/min for β -glucosidase degradation of linamarin, prunasin and taxiphylin. The K_m/V_{max} values of 0.04-0.069 dm³/min reported in this study for the native linamarase are within the range observed for isoenzymes and extracted and evaluated by Nok and Ikediobi (1999).

CONCLUSION

β -glucosidase engineered from *Saccharomyces cerevisiae* a yeast normally associated with ethanol and CO₂ production is also active in the degradation of cassava linamarin. Ion-exchange chromatographic purification techniques enhanced enzyme activity at tolerance pH (3.5-10.5) with optimum pH 6.8 and temperature 35°C. The engineered linamarase has optimum activity which increased with increased degree of purification. The enzyme purity and pH dependency of the enzymic degradation of linamarin at ambient temperature 35°C was adequately described using the Michaelis-Menten and LineWeaver-Burk kinetic models. The use of the engineered β -glucosidase is recommended for processing of cassava-based food commodities that require the use of high yielding bitter cassava varieties containing high amounts of toxic linamarin. The use of the engineered β -glucosidase can boost transformation and development of cassava-based food products for domestic human and animal consumption thereby contributing to global food security and nutrition. It can also increase the income earnings of countries due increased cassava consumption and use in food processing as well as local and international trade.

REFERENCES

Chulavatnatol, M., 2008. Characterization of β -glucosidase from Lima beans (*Prunus lunatus*). Arch. Biochem. Biophys., 265: 260-263.

Doungkamol, B., S. Thakon, A. Dumrongkiet, K. Palangpon and S. Jisnuson, 2006. β -glucosidase catalyzing specific hydrolysis of an iridoide β -glucoside from *Plumeria obtusa*. Acta Biochim. Biophys. Sin, 38: 563-570.

Flores, D.M., V.V. Garcia, M. Kojma, F. Ynagida and T. Hasegawa, 1992. Partial purification and characterization of *Aspergillus oryzae* β -glucosidase. J. Japan Soc. Food Sci. Technol., 39: 1038-1044.

Gupta, C.B., 1979. An Introduction to Statistical Methods. 8th Edn., G. Vikas Publishing House, PVT Ltd., New Delhi, pp: 424-480.

Ikediobi, C.O. and E.C. Ogundu, 1985. Screening of some fungal isolate for linamarase production. Niger. Food J., 3(1, 2 & 3): 165-167.

Ikediobi, C.O. and E. Onyike, 1987. Linamarase activity and detoxification of cassava (*Manihot esculenta*) during fermentation for garri production. Agric. Biol. Chem., 46: 1667-1670.

Keresztessy, Z., L. Kiss and M.A. Hughes, 1994. Investigation of the active site of the cyanogenic beta-D-glucosidase (linamarase) from *Manihot esculenta* Crantz (Cassava). I. Evidence for an essential carboxylate and reactive histidine residue in a single catalytic centre. Arch. Biochem. Biophys., 314: 142-152.

Krammer, A. and B.A. Twigg, 1970. Quality Control for the Food Industry. 3rd Edn., The Avi Publishing Co., Inc., Westport Connecticut, pp: 155-205.

Kuroki, G.W. and J.E. Poulton, 1986. Comparison of kinetics and molecular properties of two forms of amygdalin hydratase from black cherry (*Prunus serotina* Ehrh.) seeds. Arch. Biochem. Biophys., 247: 433-439.

Legras, J.L., M.R. Kaakeh, A. Arnaud and P. Galzy, 1989. Purification and properties of the β -glucosidase from a nitile hydratase-producing *Brevibacterium* sp. Strain R312. J. Basic Microbiol., 29: 655-669.

Lionel, V., M. Czjzek, J. Moriniere, D.R. Bevan and A. Esen, 2008. Mutation and structural analysis of aglycone specificity in maize and sorghum β -glucosidases. J. Biol. Chem., 278: 25055-25062.

Mach, R.L. and S. Zeilinger, 2009. Regulation of gene expression in industrial fungi: *Trichoderma*. Appl. Microbiol. Biotechnol., 60: 515-522.

Nok, J.N. and C.O. Ikediobi, 1999. Some properties of linamarase from cassava (*Manihot esculenta* Crantz) cortex. J. Food Biochem., 14: 477-489.

Okafor, N. and A.O. Ejiofor, 1990. Rapid detoxification of cassava mash fermenting for garri production following inoculation with a yeast simultaneously producing linamarase and amylase. Proc. Biochem., 90: 82-86.

Onyike, E., A. Ukoha and C.O. Ikediobi, 2001. Isolation and characterization of linamarase from dried cassava (*Manihot esculenta* Crantz) cortex. Niger. J. Biochem. Molecul. Biol., 16(3): 739-785.

Petruccioli, M., P. Piccioni and F. Federici, 1999a. Glucose oxidase overproduction by the mutant strain M-80.10 of *Penicillium variable* in a benchtop fermenter. Enzyme. Microb. Tech., 21: 458-462.

- Petruccioli, M., R. Brimer, A.R. Cicalini, V. Pulci and F. Federici, 1999b. Production and properties of the linamarase and amygdalase activities of *Penicillium aurantiogrisum* P35. *Biosci. Biotechnol. Biochem.*, 635: 805-812.
- Van-Boekel, M.A., 1996. Statistical aspect of kinetic modeling for food science problems. *J. Food Sci.*, 61(3): 477-485.
- Voet, D. and J.C. Voet, 1995. *Biochemistry*. 2nd Edn., Wiley Wimpenny, New York.
- Yeoh, H.H., 2009. Kinetic properties of β -glucosidase from cassava. *Phytochemistry*, 28: 721-724.