



Effects of Media Composition on the Production of Linamarase from *Lactobacillus delbrueckii* NRRL B-763

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ABSTRACT

Nutritional factors relating to the production of linamarase from *Lactobacillus delbrueckii* NRRL B-763 were investigated. The microorganism was cultivated in a medium containing 1% linamarin. Enzyme was produced using a variety of carbon substrates but the highest enzyme activity was detected in the presence of salicin (522 U/ml) after 48 h while the lowest yield was observed with carboxyl methyl cellulose (38 U/ml) after 72 h. Enzyme was not produced in the presence of cellobiose. Among a variety of nitrogen substrates tested, peptone supported maximum enzyme production (412 U/ml) after 48 h. Lowest enzyme production was observed with urea (40 U/ml). Organic nitrogen substrates generally supported higher enzyme productivity than inorganic nitrogen substrates. Enzyme activity was observed in the presence of Mn^{2+} (% relative activity = 216) while Hg^{2+} was inhibitory (% relative activity = 28). Locally - formulated media were comparable to MRS broth in supporting linamarase production by the bacterium. Higher enzyme activity was produced in media with surfactant than in media without surfactant. The enzyme may be useful in enhanced degradation of cassava cyanide.

Keywords: linamarase, locally formulated media, carbon substrates, nitrogen substrates, metal ions

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important food crop and because of the presence of cyanogenic glucosides, cassava is potentially toxic to human populations that rely on cassava-based diets. Cassava tissues contain large amounts of cyanogenic glucosides, linamarin (96%) and lotaustralin (4%) [1]. Chronic cyanide exposure associated

with the consumption of cassava has been related with a number of cyanide induced disorders including goiter, dwarfism and tropical ataxic neuropathy [2]. The establishment of a correlation between endemics of the human central nervous system syndrome *konzo* and prolonged intake of cassava products containing

residual cyanogenic glucosides has focused attention on cassava detoxification [3, 4, 5].

β -Glucosidases catalyze the hydrolysis of β -glucosidic linkages of conjugated monoglycosides [6]. A specific β -glucosidase (E.C. 3.2.1.21), linamarase (linamarin - β - D - glucoside glucohydrolase) catalyses the hydrolysis of linamarin (2-hydroxyisobutyronitrile- β -D-glucopyranoside) and a related compound lotaustralin (2-hydroxy-2-methylbutyronitrile- β -D-glucopyranoside) to release cyanohydrins which are spontaneously or enzymatically broken down to release hydrocyanic acid (HCN) which is the toxic factor in cassava [7]. Endogenous linamarase of cassava could not permit complete detoxification of cassava cyanide because of insufficient cleavage of cyanogenic glucosides of cassava or due to an inadequate maceration of the plant tissue or to an insufficient concentration of endogenous linamarase [8]. A possible solution to this problem might be the addition of exogenous linamarase during detoxification process [9]. This present investigation reports the effects of some nutritional factors on the production of linamarase from *Lactobacillus delbrueckii* NRRL B - 763.

MATERIALS AND METHODS

Microorganism and Cultivation

Conditions

Lactobacillus delbrueckii NRRL B-763 was kindly provided to the second author by Mr. James Swezey, ARS Culture and Patent Culture Collections Peoria, Illinois, USA. This organism was selected because of its high growth in a medium containing 800 parts per million KCN. Inoculum was prepared from a stock culture by transferring to an Erlenmeyer flask (250 mL) containing 100 mL of medium A with the following composition (w/v): NaCl (BDH), 0.5%; peptone (Oxoid), 0.5%; lactose (BDH), 1%;

linamarin (BDH), 1%. The medium was sterilized at 121°C for 15 minutes. The inoculum was grown for 24 h in a Gallenkamp orbital incubator at 100 $\times$ g at 30 $\pm$ 2°C. The cells were collected by centrifugation at 2515 $\times$ g for 15 minutes, washed twice with 0.2 M phosphate buffer (pH 6.5) and diluted to an optical density of 0.1 measured in a Spectrumlab 23A spectrophotometer at 600 nm.

The effects of carbon sources on linamarase production was determined by incorporating various carbon sources- soluble starch (May and Baker), salicin (BDH), CM cellulose (BDH), sucrose (Matheson Coleman and Bell), glucose (BDH), maltose (BDH) and cellobiose (BDH) at 1% (w/v) concentration into 100 ml of medium A devoid of lactose.

To investigate the effects of nitrogen sources on linamarase production, 0.5% (w/v) of various nitrogen sources namely skim milk (Oxoid), tryptone (Oxoid), yeast extract (Difco), casein (Difco), ammonium sulphate (BDH), potassium nitrate (May and Baker) and urea (BDH) were added into 100 ml of medium A in place of peptone.

The effect of metal ions on enzyme activity was investigated by incorporating 1 mM solutions of various salts into medium A.

Preparation of Broths

Tomato Juice Broth (TJB)- Fresh tomato fruits (200 g) were homogenized in a blender containing 1 L of deionized water and filtered with a stainless steel mesh. The filtrate was re-filtered with a Whatman No. 1 filter paper. A 1% linamarin (w/v) was added into the medium (pH = 4.7) which was sterilized by tyndallization according to Collins and Lyne [10].

Orange Broth (OB)- Peeled fully-ripped oranges (200 g) were ground with a Corona mill (Medellin, Colombia) after removing the

seeds in 1 L of deionized water and filtered with a stainless steel mesh. The filtrate was re-filtered with a Whatman No. 1 filter. A 1% (w/v) linamarin was added into the medium (pH = 4.6) which was sterilized by tyndallization.

Banana Broth (BB)- Ripe banana fruit (200 g) was homogenized with mortar and pestle in 1 L of deionized water and filtered with a stainless steel mesh. The filtrate was re-filtered with a Whatman No. 1 filter. A 1% (w/v) linamarin was added into the medium (pH = 5.8) which was sterilized by tyndallization.

Tomato juice broth, orange broth, banana broth and a modified MRS (Man, Rogosa and Sharpe) broth containing 1% (w/v) linamarin were each dispensed in 100 ml amounts into sterile conical flasks and were tested for their ability to support enzyme production by growing the culture in the media. The effects of Tween 80 on the production and activity of the enzyme were studied by adding 0.1% (w/v) Tween 80 (Difco, Laboratories, USA) into TJB, BB, OB and modified MRS broth at the time of incubation.

Extraction of Linamarase

Cells were grown and harvested by centrifugation at $2515 \times g$ for 15 minutes and washed twice with 0.2 M phosphate buffer (pH 6.5). The washed cell suspension was disrupted by sonication for 10 min. using Biologics Ultrasonic homogenizer Model 150VT (115V/60Hz). Following disruption, the mixture was centrifuged at $2515 \times g$ for 15 minutes. The supernatant fluid was used to measure enzyme activity.

Assay Procedures

Protein content was estimated by the method of Lowry *et al.*, [11] using bovine serum albumin (Sigma-Aldrich) as a standard.

Cyanide was determined by a modification of the alkaline picric acid method of Williams and Edwards [12] as follows: various quantities of standard (50, 100, 150 and 200 $\mu\text{g/ml}$) solution of KCN were added into tubes containing 2 ml of 2% KOH and 1ml of picric acid: Na_2CO_3 : H_2O (1:5:200 v/w/v). The tubes were incubated for 10 minutes in a 37°C water bath, cooled for 20 minutes in a refrigerator and read in a Spectrum lab 23A spectrophotometer at 510nm. The readings were used to draw a standard curve for micrograms KCN per ml against absorbance.

Linamarase activity was assayed by determining the HCN liberated from linamarin as follows: 0.5 ml of enzyme solution in 0.2 M phosphate buffer (pH 6.5) contained in Eppendorf tubes was added to 0.5 ml of 1 mM buffered (same buffer) solution of linamarin (BDH, Poole, England). After 20 minutes of incubation at $32 \pm 2^\circ\text{C}$, 2 ml of 2% KOH and 1 ml of picric acid: Na_2CO_3 : H_2O (1:5:200 v/w/v) were added into the reaction mixture. The reaction was stopped by placing the tubes in iced water. The red colour that developed was read at 510 nm in a spectrophotometer. Under the above conditions, one unit of activity was defined as the amount of enzyme that released 1 μg HCN in 30 minutes under the assay condition.

Results and Discussion

Results in Table 1 show the influence of different carbon sources on linamarase production by *Lactobacillus delbrueckii* NRRL B-763. The carbon sources were each incorporated into the growth media at 1% (w/v) concentration as sole carbon sources in place of lactose. Beneficial effects of lactose on linamarase production was reported by Okafor and Ejiofor [13]. Enzyme production in most cases reached

a peak at 48 h and decreased thereafter. This may be associated with the onset of the stationary phase of microbial growth during which microbial metabolite synthesis is highest [14]. With the exception of cellobiose, the bacterium grew and produced enzyme in all media tested. Substitution of a variety of carbon sources showed that highest enzyme activity was produced when the organism was grown in a medium containing salicin

as the sole carbon source (522 U/ml) after 48 h. Low enzyme activity was produced when soluble starch and CM cellulose were used as sole carbon sources (Table 1). The organism did not produce linamarase activity when cellobiose was used as a sole carbon source. This finding is similar to data relating to a β -glucosidase [15] which did not hydrolyze cellobiose at concentrations of 0.1-8.0 mM.

Table 1. Effects of carbon sources on the production of linamarase by *Lactobacillus delbrueckii* NRRL B-763.

Carbon source (1%, w/v)	Period of incubation (h)		
	24	48	72
	Linamarase activity (Unit/ml)		
Cellobiose	0 $\pm$ 0.0	0 $\pm$ 0.0	0 $\pm$ 0.0
CM cellulose	19 $\pm$ 2.8	42 $\pm$ 8.4	38 $\pm$ 1.4
Glucose	232 $\pm$ 2.8	358 $\pm$ 7.1	144 $\pm$ 4.3
Lactose	228 $\pm$ 4.3	354 $\pm$ 4.2	139 $\pm$ 5.7
Maltose	315 $\pm$ 8.4	242 $\pm$ 8.5	133 $\pm$ 4.2
Salicin	386 $\pm$ 4.2	522 $\pm$ 8.5	404 $\pm$ 2.8
Soluble starch	112 $\pm$ 8.5	118 $\pm$ 2.9	76 $\pm$ 7.1
Sucrose	328 $\pm$ 4.2	247 $\pm$ 5.7	234 $\pm$ 2.8

Mean $\pm$ SD

Values are significantly different (≤ 0.05)

The effect of various nitrogen sources on the production of linamarase is shown in Table 2. The highest enzyme activity was produced when organic nitrogen substrates were used to grow the organism. Enzyme activity was highest (412 U/ml) in medium containing peptone as the sole nitrogen source after 72 h. Comparatively, inorganic nitrogen sources caused a lower linamarase production by the organism with urea producing 40 U/ml after 72 h (Table 2). Findings on other β -glucosidases indicated that organic

nitrogen sources were preferred nitrogen substrates for enzyme production and activity. Mahajain *et al.*, [16] screened different nitrogen sources for their effect on α - and β -glucosidase production. Among ten nitrogen sources tested, highest level of α - and β -glucosidase was obtained when organic nitrogen substrates were used. Chan and Li [17] also reported that organic nitrogen sources produced higher levels of α -glucosidase.

Table 2. Effects of nitrogen sources on the production of linamarase by *Lactobacillus delbrueckii* NRRL B-763.

Nitrogen source (0.5%, w/v)	Period of incubation (h)		
	24	48	72
Linamarase activity (Unit/ml)			
Organic			
Casein	225±5.7	296±1.4	242±1.4
Peptone	308±5.6	412±4.2	344±4.9
Skim milk	288±2.8	306±8.5	268±2.8
Tryptone	296±4.2	317±4.2	274±4.2
Yeast extract	208±8.5	296±4.2	244±7.1
Inorganic			
Ammonium sulphate	112±7.1	226±8.5	188±1.4
Potassium nitrate	88±4.2	96±2.8	58±2.8
Urea	72±9.9	68±1.4	40±4.2

Mean ±SD

Values are significantly different (≤ 0.05)

The effects on enzyme activity of various metal ions were investigated (Table 3). The activity was strongly inhibited by the addition of Hg^{2+} at a concentration of 1 mM (% relative activity = 28). Highest activity was obtained with Mn^{2+} (% relative activity = 216). Watanabe *et al.*, [18] reported the inhibition of cyanide - hydrolyzing enzyme from *Pseudomonas stutzeri* AK6 by Hg^{2+} suggesting a possible presence of thiol groups at the catalytic

site of the enzyme. Esen [19] also reported that Hg^{2+} was a potent inhibitor of β -glucosidase activity. Meyers *et al.*, [20] reported that Mn^{2+} was specifically required in growth media for the production of cyanide - degrading activity of *Bacillus pumilus*. The authors concluded that Mn^{2+} was required either for induction of the gene(s) controlling the cyanide - degrading enzyme activity or as a component of the cyanide-degrading enzyme system.

Table 3. Effects of metal ions on the production of linamarase by *Lactobacillus delbrueckii* NRRL B-763.

Metal salt (1 mM)	Specific activity (Unit/mg protein)	% Relative activity
None	82.4±0.7	100
AgNO_3	103.2±2.3	125
$\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$	128±4.2	155
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	166.3±5.2	202
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	106±5.7	129
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	96±4.2	117
HgCl_2	23.2±0.6	28
MnCl_2	178±2.8	216
$\text{Pb}(\text{NO}_3)_2$	33±4.2	40
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	88±3.5	107

Mean ±SD

Locally - formulated media namely, banana broth, orange broth and tomato juice were comparable to MRS broth in promoting linamarase production by the organism. Addition of vegetable extracts which are cheap and readily available to a growth - promoting medium for subsequent enhancement of microbial growth and activity has been employed in many laboratories [21]. The microorganism grew and produced linamarase in all the media (Table 4). Incorporation of Tween 80 at 0.1% (w/v) concentration at the time of inoculation significantly (≤ 0.05) increased enzyme productivity by the organism (Table 4). The explanation was that the surfactant acted on the cell membrane to facilitate the release

of the enzyme into the medium. Surfactants are substances which accumulate at surfaces and interfaces [22]. Demain and Birnbaum [23] found that surfactants alter cell membrane permeability thereby facilitating secretion of the enzyme into the growth medium. The stimulatory effects of Tween 80 on bacterial enzyme production have been reported by many researchers [24, 25, 26, 27]. Reese *et al.*, [22] and Reese and Maguire [28] showed that addition of certain surfactants to fungal cultures increased enzyme production. Abalaka and Garba [29] reported that the addition of Triton X-100 and Tween-80 increased fungal growth and linamarase production probably because of cell membrane damage.

Table 4. Production of linamarase by *Lactobacillus delbrueckii* NRRL B-763 in various media.

Media	Period of incubation (h)			Period of incubation (h)		
	24	48	72	24	48	72
	Linamarase activity (Unit/			Linamarase activity (Unit/ml)		
	Control			0.1% Tween 80 added at inoculation		
Banana broth	75±2.8	186±5.6	92±1.4	341±1.4	389±8.5	352±4.2
MRS broth	251±4.3	296±2.9	206±1.4	537±7.1	592±2.8	564±3.6
Orange broth	180±4.2	206±8.5	175±2.8	312±4.2	366±5.7	294±3.5
Tomato juice broth	177±2.8	288±4.2	208±4.3	520±8.5	588±4.2	497±4.9

Control: Without Tween 80 solution

Mean ±SD

Values are significantly different ($p \leq 0.05$)

CONCLUSION

This work shows the effects of some media components on the production of linamarase enzyme by *Lactobacillus delbrueckii* NRRL B-763. Agro-based substrates were comparable to some chemically - defined media for enzyme production by the bacterium. The addition of surfactants into the basal media increased enzyme productivity.

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