IN VITRO STUDIES OF CELLULOSE DIGESTING PROPERTIES OF *STAPHYLOCOCCUS SAPROPHYTICUS* ISOLATED FROM TERMITE GUT

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ABSTRACT

Staphylococcus saprophyticus inhabiting the gut of *Odontotermes obesus* is a potential cellulose deploymerizer. The cellulase activity (both C_x and C_1) was extracellular and was mainly located in the culture supernatant. As the culture ages, the cellulose is consumed and the bound enzyme is released to the culture fluid. Maximum activity was recorded when the concentrations of yeast extract and the CMC in the incubation medium were 0.6% and 1.5%, respectively. The pH and temperature optima for depolymerization of cellulose were 6.5 and 45°C respectively.

INTRODUCTION

TERMITE gut microbiota presents an unique biological system for the study of the conversion of woody and cellulosic substrates into useful products through the process of solubilization. Information is scanty regarding the physiological and biochemical aspects of the gut microbiota of higher termites—*Termes* (Cyclotermes) *obesus* also known as *Odontotermes obesus* which are devoid of flagellates and protozoan fauna and is one of the most prevalent species occurring in the semidesert soils of Arravali hills, Delhi and Rajasthan. Several authors have studied[1-3] the enzyme cellulase (namely C_1 and C_x) in the whole gut preparation of the termite and attributed the activity to the cellulose degrading bacteria inhabiting the gut. Later Thayer[4] isolated several facultative cellulose digesting bacteria from the gut of *Reticulitermes hesperus*; however, the existence of cellulolytic bacteria from the gut was doubted by O'Brien and Slaytor[5]. Thus it is now an open question whether higher termites in general possess microbial spectrum with full complement of enzymes[6] required to digest native cellulose. This communication relates to *in vitro* characterization of cellulose solubilization by *Staphylococcus saprophyticus*.

MATERIALS AND METHODS

S. saprophyticus was isolated from the hind gut of termite *O. obesus*. The culture was grown in nutrient broth in Erlenmeyer flask on a rotary shaker (240 r.p.m.)[7] at 32°C. The growth rate was determined by measuring the total protein[8]. Cellulose-grown cultures (late exponential phase) were first centrifuged (Sorvall RC 5) for 30 min at 1250 g to remove cellulose. The cellulose collected from the centrifuged culture medium (30 S at 1250 g) was washed 3–4 times by decanting in an ice-cold buffer solution containing 45 mM of Na_2HPO_4; 70 mM of NaCl; 20 mM of KH_2PO_4 and 0.5 mM of $MgSO_4$ (pH 7.0). This removed free enzymes and most of the remaining bacteria and used as fraction I. The cellulolytic activity bound superficially to the wall of the residual bacteria was removed by suspending the cell paste in the buffer and subjected to gentle shaking for 20 min using a magnetic stirrer. The cell washings served as fraction II. The residual cells resuspended in the buffer and were subjected to ultrasonic disintegration for 2 min (1 minute bursts followed by 30 sec gaps, to avoid heating of samples) at an amplitude of 12 kHz using a MSE ultrasonic disintegrator fitted with a titanium probe. The sonicated mixture was clarified by centrifuging at 15,000 g for 10 min. The supernatant served as fraction III. Cell debris, broken walls and membranes were suspended in the buffer and used as fraction IV. Fractions were dialyzed overnight with three changes of the same buffer before use.

The C_x-activity (activity towards carboxymethyl cellulose) was determined following the procedure of Berg *et al*[9]. An aliquot of enzyme sample (0.3 ml) was mixed with 2 ml of substrate (CMC solution) and incubated at 40°C for 20 min. The reaction was stopped by adding 3 ml of DNSA (dinitrosalicylate reagent) and the mixture boiled in a water bath for 15 min. Colour was measured at 640 nm after cooling. One milliunit (mU) of cellulase was defined as the amount of enzyme which could release 1 μ equivalent of glucose from CMC or microcrystalline cellulose/min.

The microcrystalline cellulose degrading activity (C_1) was measured after the method of Somogyi[10]. An aliquot of enzyme sample 0.5 ml was added to 2 ml of 1% suspension of sigmacell in 0.05 M PO_4 buffer,

pH 7.0, and incubated on a rotary shaker for 24 hr at 30°C. The reaction was stopped by adding 2 ml of copper reagent and the suspension was filtered to remove fibres. The filtrate was boiled for 15 min, cooled and 2 ml of Nelson chromogen was added. The sample was then allowed to stand at room temperature for atleast 20 min, filtered through a filter paper and the colour was measured at 510 nm. A standard curve was established with glucose. Fractionation of enzyme was carried out in a cold room at 4°C. To determine the influence of concentration of yeast extract and carboxymethyl cellulose to free and cellulose bound activities, the cells were grown with shaking at 32°C. Samples were chilled and cellulase fractions prepared as described above and assayed for the activity. The cultures were incubated up to the late exponential phase until the activity was no longer detectable in the medium. The pH values of the culture broth were adjusted with 1 N NaOH and HCl and controlled before and after the experiment. For enzyme assays the incubation mixture was adjusted from pH 5 to 9 using sodium citrate and phosphate buffer. To determine the optimum temperature, the reaction mixture preincubated at the desired temperature and then the enzyme fraction were included to initiate the hydrolytic reaction. All experiments were duplicated. The isolate was identified using Bergey's manual[11].

All the chemicals were bought from Sigma Chemicals, USA except the yeast extract and peptone which were from Difco, Michigan and CMC cellulose from BDH, England.

RESULTS

Isolate is a facultative aerobic, non-motile, non-spore forming, gram-positive coccoid (0.7 μm in diam), catalase-positive and coagulase-negative, arranged in irregular manner. Cells are high sodium chloride tolerant (about 10% and above) and G + C content, 37 moles% (T_m). In log phase, fermented mannitol and metabolized glucose the end product was acetoin; reduced nitrate to nitrite; Voge's Proskauer test negative and hydrolyzed gelatin gradually.

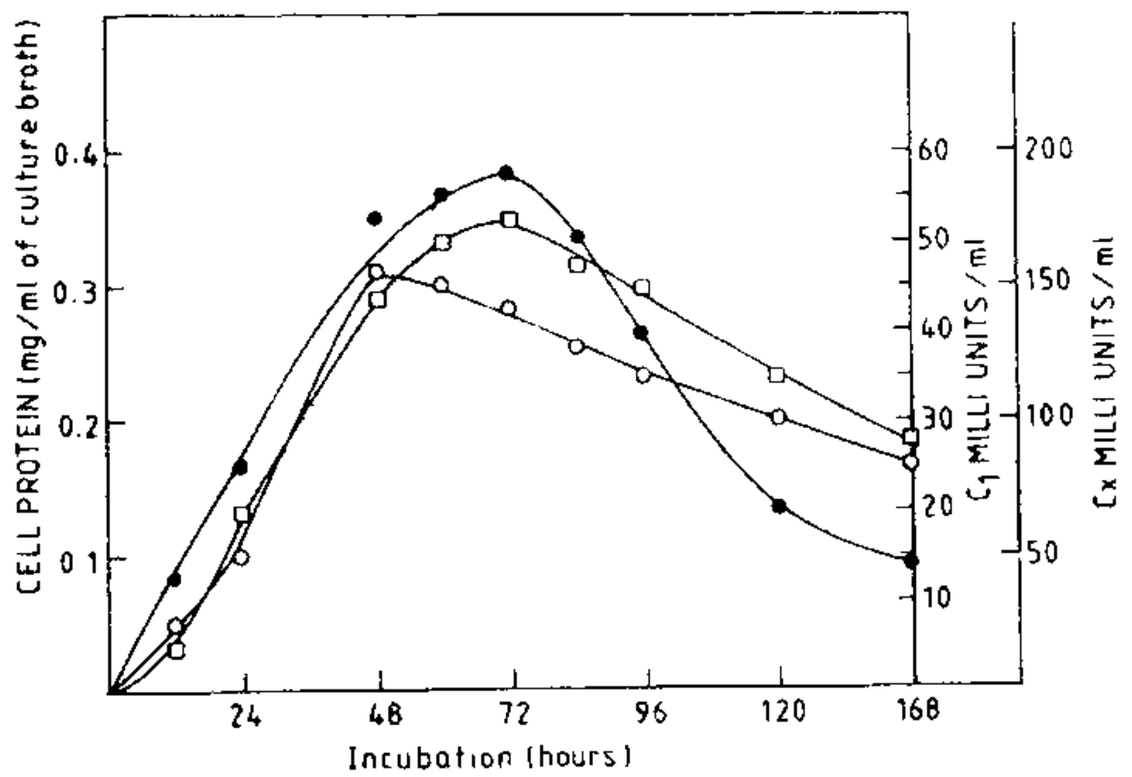

Figure 1. For growth and CMC hydrolyzing (C_x) and Sigmacell hydrolyzing enzyme activity (C_1) of *Staphylococcus saprophyticus*, cells were grown in broth culture containing (g/l) CH_3COONa, 3.0; Na_2SO_4, 7 H_2O, 0.4; $MgSO_4$, 7 H_2O, 0.2 $MgCl_2$, 6 H_2O, 1.8; K_2HPO_4, 0.25; KH_2PO_4, 0.25; $CaCl_2$, 2 H_2O, 0.2· $FeSO_4$, 0.01; EDTA, 0.04. After autoclave, yeast extract (0.6%) and CMC (1.5%) sterilized separately were aseptically added. Final pH was adjusted to 7.3, □ —— □ Cell protein; ●——● C_x and o——o C_1 hydrolyzing estimation.

Carboxymethyl cellulase was produced maximally in the late exponential or stationary phase of growth while growing on 1.5% CMC and 0.5% yeast extract (figure 1). The enzyme activity was primarily localized in the cell-free culture filtrate (fraction I) and very low amounts in the cell washing (fraction II). Negligible depolymerization of cellulose was located in the cytoplasm (fraction III) and cell wall and membranes (fraction IV) (table 1). The optimum depolymerization

Table 1 *Localization of cellulase activity*

Fractions	C_x^*		C_1^*	
	Mid exponential	Early stationary	Mid exponential	Early stationary
I	181	190	47	43
II	23	27	5	12
III	11	15	nil	4
IV	4	3	nil	nil

*activity expressed in mμ/ml.

of CMC was obtained at 0.6% yeast extract in the incubation broth (table 2). Further addition exerted an adverse effect on cellulase production. Yeast extract was essentially required for the growth and cellulase production and could not be replaced by Difco Casamino acid, Difco Bactopeptone, Difco Bactocasitone and Vitamin solution. CMC (1.5%) promoted optimum enzyme production. Any further addition had an inhibitory effect on enzyme; however, the cell protein had attained a steady state of growth up to 3% CMC (table 3). Optimum pH for cell yield was 7.3 (figure 2). When the reaction mixtures were buffered with sodium citrate or potassium phosphate buffer, maximum enzyme activity was recorded at pH 6.5 (figure 3). In general C_x activity showed broader pH tolerance than C_1 activity. An Arrhenius plot of growth rate (figure 4) indicated that ideal temperature for

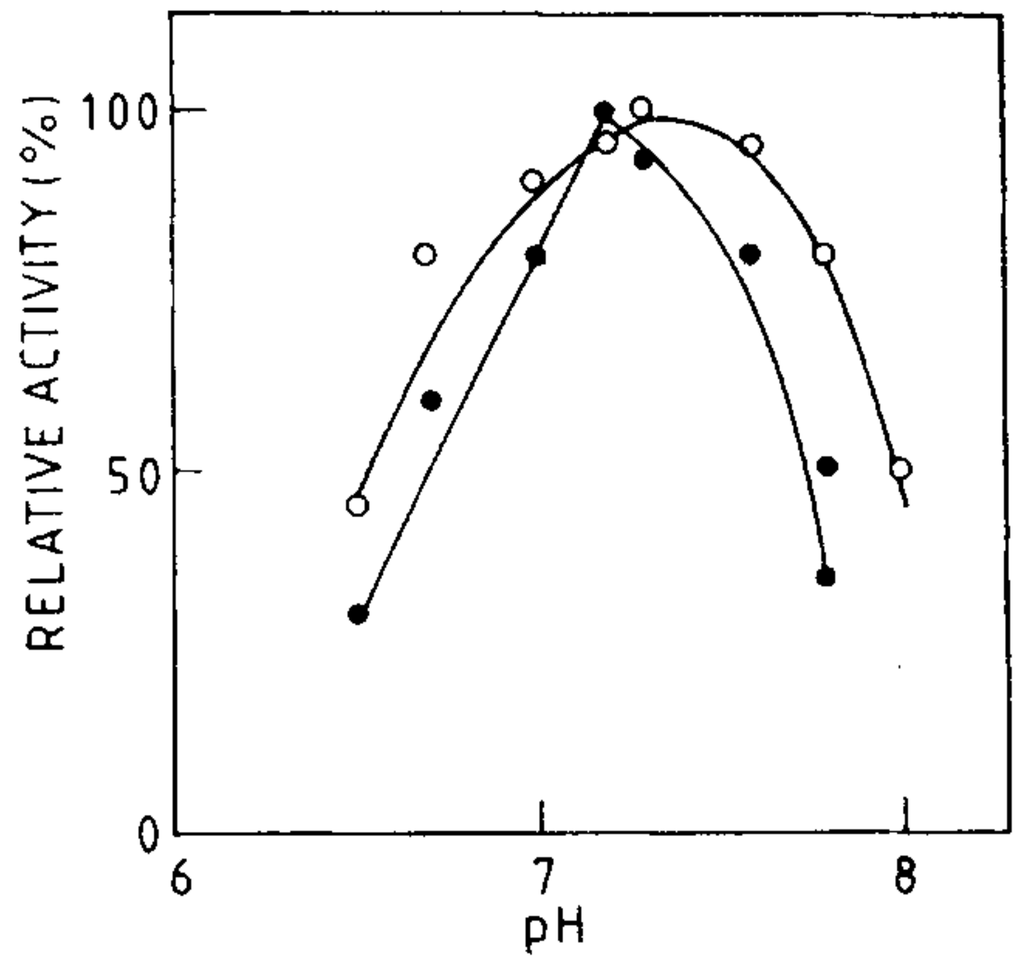

Figure 2. Effect of pH on cellulase production. The initial pH of the medium was adjusted with phosphate buffer (Final concentration, 0.1 N) ●——● C_x production. o——o C_1 production.

Table 2 *Effect of yeast extract concentrations on growth and cellulase production (C_x-CMC) hydrolyzing activity, C_1 Sigmacell hydrolyzing activity*

Yeast extract %	C_x	C_1	Protein (mg/ml)
Nil	9	2	0.08
0.1	74	10	0.16
0.2	90	13	0.20
0.3	98	20	0.26
0.4	112	27	0.32
0.5	119	30	0.40
0.6	132	32	0.48
0.7	47	16	0.28
0.8	32	8	0.26
1.0	25	4	0.23

*Activity expressed as mμ/ml.

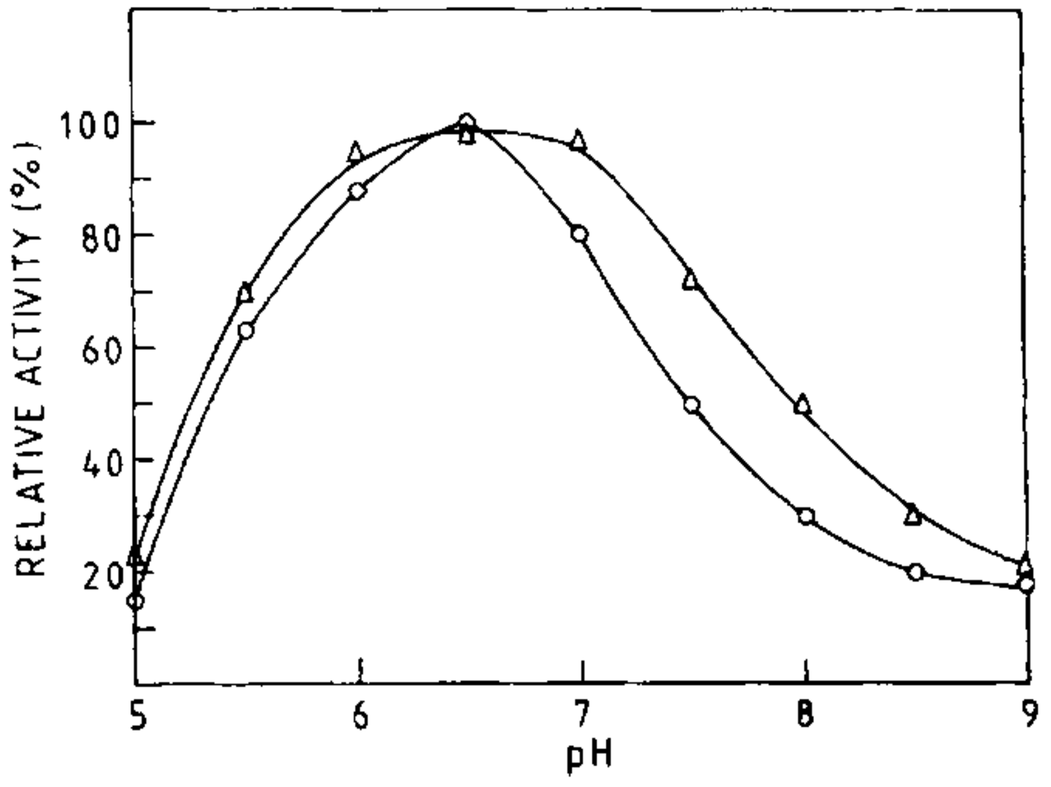

Figure 3. Effect of pH on cellulase activity. A culture from late exponential growth phase was taken. Reaction mixtures were buffered with 0.1 M sodium citrate buffer (pH 5.0 to 6.0) or with 0.05 M phosphate buffer (pH 6.0 to 9.0) Δ C_x activity O C_1 activity.

Table 3 *Effect of cellulose concentration (CMC) on growth and cellulase production*

CMC %	C_x^*	C_1^*	Protein (mg/ml)
0.1	114	8	0.14
0.5	117	26	0.25
1.0	156	28	0.35
1.5	178	48	0.61
2.0	159	36	0.63
3.0	153	34	1.21

*Activity expressed as mμ/ml.

growth of *S. saprophyticus* on minimal medium containing 0.6% yeast extract and 1.5% CMC was between 30° and 36°C with a maximum at 32°C. The temperature at which the bacterium caused the highest rate of depolymerization of CMC was 45°C. Enzyme preparation was sufficiently stable up to 50°C beyond which a sharp decline was recorded.

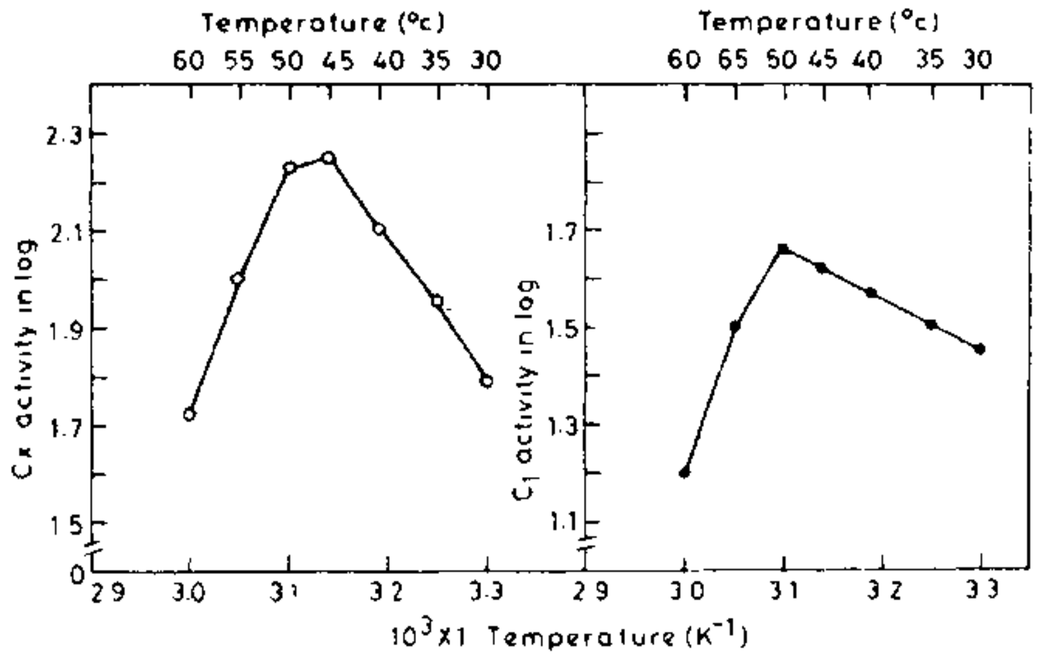

Figure 4. Effect of temperature on cellulase activity. Arrhenius plot of the rate of o——o C_x and ●——● C_1 activity with temperature. Measurements were made at 5°C intervals from 30°C to 60°C.

DISCUSSION

The nutritive metabolism of termites is based upon the exploitation of cellulosic materials. In the lower termites (families Mastotermitidae, Kalotermitidae, Hodotermitidae, Rhinotermitidae and Serritermitidae), cellulose is digested by protozoa residing in the paunch of the hind gut[12]. The members of higher termite family Termitidae lack this assemblage of xylophagous protozoa, and the mechanism by which the ingested cellulose is degraded remains largely a matter of speculation. It has been suggested that in some species paunch bacteria assumed the role of the protozoa but evidence in support of this notion is meagre[5]. Cellulases have been reported in the gut of several species of higher termites[1-3, 13-17]. Thayer[18] isolated the faculative wood-digesting bacteria, *Bacillus cereus, Serratia marcescens, Arthrobacter* and *Alcaligens* sp from the gut of *Reticulitermes hesperus. In vitro* studies on *Bacillus* and *Serratia* sp, under aerobic incubation have shown the presence of CMC-hydrolyzing enzyme[4], which was disputed by O'Brien and Slaytor[5], on the ground that the bacteria were transient organisms ingested by the termite and fail to accept them as autochthonous flora because of the anaerobic nature of the hind gut. In a recent study[7] on termite mound soil we have reported predominantly the presence of rod-shaped bacteria and the gut bacteria which were by and large coccoid except one species of *Bacillus.*

All the gut isolates were facultative in nature and these strains failed to appear on the enrichment cultures of mound soils. Our findings agree with that of Thayer[18] and provide evidence that *O. obesus* most frequently encountered in the desert and semi-arid regions of India are devoid of protozoa, and harbour active cellulose-digesting bacteria in the gut system and thereby derives energy by the solubilization of cellulosic materials ingested by the termites. Results from our laboratory[7, 19] further suggest that cellulosic materials are solubilized to a great extent by aerobic soil-inhabiting microbes being ingested by the termite. The gut microflora further solubilizes the undigested cellulosic materials to glucose and low carbon compounds[7] with the mediation of facultative coccoid gut bacteria.

C_x type of cellulases have been demonstrated widely by several authors but C_1 type has been described only in gut of *Trinervitermes trinevoides*[13]. This finding recorded the production of C_1 cellulase although the activity was low (48 mU/ml) as compared to C_x activity (197 mU/ml). Lee and Blackburn[20] in a thermophilic *Clostridium* sp and Berg *et al*[9] in a cellulolytic bacterium *Cellvibrio fulvus* observed extracellular cellulase whereas Thayer[4] observed both cell-free and cell-bound activities in *Bacillus cereus* and only cell bound in *Serratia marcescens.* In the present study both C_1 and C_x cellulases were produced extracellularly constituting most of the extracellular proteins in actively growing cultures; this observation is in conformity with the earlier observations[21] in *Ruminococcus albus.* Although the isolate is mesophile, the crude enzyme preparations were sufficiently stable up to 50°C. This observation is in line with the alkalophilic *Bacillus* strain isolated by Horikoshi *et al*[22].

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