0273-2289/92/3435—0317$02.60

Use of Chemostat for Enhanced Production of β-Glucosidase by Newly Isolated Anaerobic Cellulolytic *Clostridium* Strain RT9

R. TABASSUM,* M. I. RAJOKA, AND K. A. MALIK

Biotechnology Laboratory,
Nuclear Institute for Agriculture and Biology,
P.O. Box 128, Faisalabad, Pakistan

ABSTRACT

A new anaerobic, mesophilic, spore-forming, cellulolytic *Clostridium* strain, RT9, was isolated from bovine rumen fluid. This strain has the ability to hydrolyze cellulose (Sigma cell-100), carboxymethylcellulose (CMC), and cellobiose into ethanol, acetate, butyrate, lactate, acetone, H_2, and Co_2 as the major fermentation products. The bacterium also exhibited endoglucanase, exoglucanase, and β-glucosidase activities in batch culture during growth on chemically defined medium with cellulose as the sole carbon source. Chemostat experiments were conducted under pH, dilution rate, and carbohydrate limitations to study the effect of various parameters on enzymes and product formation. In chemostat with 0.5% cellobiose as the limiting nutrient, maximum β-glucosidase activity and ethanol production occurred at pH values of 5.5 and 6.5, respectively. At low pH, acid accumulation was higher. β-glucosidase production increased up to 17-fold at a low dilution rate, and ethanol raised twofold. Relative activity of β-glucosidase on a percent basis was maximum at pH 6.5 and 45°C. In a carbon-limited, continuous-culture experiment, when CMC was used as an inducer in cellobiose-growing culture, β-glucosidase activity was higher than the cellobiose-limited chemostat. On the other hand, enzyme declined twofold, and ethanol production increased in glucose limitation.

Index Entries: *Clostridium* strain RT9; rumen; cellulolytic enzymes; β-glucosidase expression; chemostat.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Production of biofuel through a photosynthetically derived substrate is a very attractive proposition, particularly for a country whose economy is based on agriculture. Pakistan has not been as lucky in its energy resources as in agricultural potential. The main source of commercial energy is natural gas, followed by hydroelectricity and a small amount of coal and oil. The anaerobic thermophile *Cl. thermocellum* has been a potential industrial organism for direct conversion of cellulose to ethanol (*1*). The cellulose complex of *Trichoderma reesi* and *Cl. thermocellum* has been studied extensively (*2,3*). The quantitative contribution of mesophilic to the anaerobic degradation of cellulosic material is much greater than that of thermophilic bacteria because of the common occurrence of mesophilic cellulytic bacteria in various natural habitats, so it is possible that a wide diversity of these organisms exists in nature (*4*). Many cellulytic *Clostridia, Cl. lochheaddi, Cl. longisporum, Cl. polysaccharolyticum,* and *Cl. cellobioparum,* have been isolated from the rumen (*5–7*). These anaerobes can play a major role in mesophilic anaerobic digestor working on lignocellulosic material for the production of biofuel. This article describes the isolation and characterization of a new clostridium strain from bovine rumen. The cellulytic enzyme system of the new isolate in batch and chemostat cultures was studied.

MATERIALS AND METHODS

Culture Medium and Isolation

The strain was isolated from rumen fluid-enriched culture. The culture was established in the laboratory on a basal medium containing cellulose as a carbon source. The basal medium had the following composition (mg/L, w/v): $NaHCO_3$, 2060; NH_4Cl, 680; K_2HPO_4, 296; KH_2PO_4, 180; $(NH_4)_2\ SO_4$, 150; $MgSO_4{\cdot}7H_2O$, 120; $CaCl_2{\cdot}2H_2O$, 61; $FeSo_4{\cdot}7H_2O$, 21; $N(CH_2COOH)_3$, 15; NaCl, 10; $MnSO_4{\cdot}H_2O$, 5; $CoCl_2{\cdot}6H_2O$, 1; $ZnSo_4{\cdot}7H_2O$, 1; $CuSO_4{\cdot}5H_2O$, 0.1; $AlK(SO_4)_2{\cdot}12H_2O$, 0.1; H_3BO_3, 0.1; $Na_2MoO_4{\cdot}2H_2O$, 0.1; Pyridoxine HCl, 0.1; Thiamine HCl, 0.05; Riboflavin, 0.05; Nicotinic acid, 0.05; Para-amino Benzoic acid, 0.05, Lipoic acid; 0.05; Biotin, 0.02; Folic acid, 0.02; Vitamin B12, 0.005; and resazurin, 1. This medium also contained 250 mg/L of cysteine-HCl and 250 mg/L of $Na_2S{\cdot}9H_2O$. The medium was supplemented with 1% cellulose (Sigma cell-100) for the isolation purpose. The medium was prepared under oxygen-free nitrogen. The strain was isolated in cellulose agar roll tubes. The colonies that showed zones of clearance in cellulose agar were picked and purified by making serial twofold dilutions in cellulose broth or cellobiose medium. Purified colonies were transferred to cellulose medium in triplicate. Incubation was carried out at 37°C.

Biochemical Tests

Biochemical tests were carried out in basal medium containing the desired substrate as described by Holdeman and Moore (*8*). For testing the utilization of substrate, cultures were incubated more than 1 wk before recording them negative. Growth was measured by reading the OD (610 nm) of a culture with a Bausch and Lomb, Spectronic 710, spectophotometer.

Morphological Characterization

The morphological properties of the strain were examined by phase-contrast microscope and by electron microscope after negative straining according to Valentine et al. (*9*). An electron micrograph was taken with a Philipps EM 301 electron microscope. Characteristics of colonies were determined on cellulose agar showing cleared zone of cellulose digestion.

Determination of DNA Base Composition

Cells (10 g) were obtained from an exponentially growing culture under continuous flushing of oxygen-free nitrogen. DNA was prepared by the method of Marmur (*10*). The mol% G+C content of DNA was determined optically by thermal denaturation using a Gilford model 2600 spectrophotometer and thermal programmer model 2527. All calculations have been made using the equation $G+C=2.44\ (Tm-69.4)$ (*11*). DNA from *E. coli* K12 (mol% $G+C=51.7$) was used as a reference.

Continuous Culture Operation

The chemostat experiments were performed in a 2-L Biostat fermenter with a 600-mL culture volume. The culture volume was maintained constant by providing an overflow pipe for gases and cell suspension. The dilution rate was regulated by the inflow of fresh medium using a peristaltic pump. Anaerobiosis was maintained by flushing oxygen-free nitrogen gas through the fresh medium and culture. The experiments were conducted at an agitation rate of 200 rpm, at a temperature of 37°C, and at different pH ranges. The pH was measured with a glass electrode and maintained at the desired value by automatic addition of 2*N* KOH.

Chemical Analysis

Fermentation products, such as ethanol, acetate, butyrate acetone, and butanol, were determined by gas chromatography. The samples were acidified with 2*N* HCl, and 1 mL was injected into a Perkin Elmer gas chromatograph equipped with a flame ionization detector. The 2-m column was packed with Chromosorb 101 (80/100 mesh). The column temperature was 150°C, injected temperature was 200°C, and the detector temperature was 220°C. Nitrogen was used as the carrier gas. The data were analyzed with Shimadzu CR2 AX integrator. Concentrations were calculated

with isobutanol as an internal standard. L (−) Lactate and D (−) lactate were determined enzymatically by using a lactate dehydrogenase-based kit (Boehringer-Mannhein Gmbh, Biochemica, W. Germany).

Enzyme Assay

For cellulytic enzyme assays, the culture supernatant or cell extract was incubated with one of the following substrates: carboxymethylcellulose (CMC Sigma company), the substrate used for determining endoglucanase activity or Whatman no. 1 filter paper for combined exoglucanase and endoglucanase activity. The hydrolysis of these substrate was measured in 0.2*M* acetate buffer (pH 5–6) at 40°C for carboxymethyl cellulase (CMCase) and at 50°C for filter paper activity. In these tests, reducing sugars were estimated calorimetrically with dinitrosalicylic acid reagent. One unit of CMCase activity is defined as 1 μg glucose equivalents released/mL/min and 1 U of filter paper activity is defined as 1 μg glucose equivalents released/mL/h. The β-glucosidase and β-xylosidase activities were measured by using the corresponding glycoside. Units are defined as μg nitrophenol produced/mL/min (*12*).

RESULTS AND DISCUSSION

Isolation and Characterization

The *Clostridium* strain designated RT9 was isolated from fistulated bovine rumen. The inoculum was enriched on basal medium supplemented with cellulose. The culture was purified after repeated heating at 80°C for 10 min, followed by serial dilution in cellulose broths. Purified colonies were picked from cellulose roll agar tubes, which appeared after 3–5 d. Colonies were minute in size and showed 2–5 mm cleared zones of cellulose digestion. Under dissecting microscope observations, colonies were white to yellowish colored, rouind, and convex with smooth edges. Complete digestion of cellulose containing large number of colonies was observed after prolonged incubation of roll tubes. Cells under phase-contrast microscope were gram-negative rods approx 0.5–8.0 μm with pointed ends occurring mostly as individuals or in pairs. The single cells were curved, spindle-shaped rods, motile, and not encapsulated (Figs. 1, 2a and 2b). Oval terminal spores were found both on cellulose and cellobiose growing media. Negatively stained cells under electron microscope revealed the presence of peritrichous flagellation (Figs. 1 and 2). The temperature range varied from 28–45°C, but the optimum temperature for growth was 37°C. The optimum pH was 6.8–7.0. Rumen fluid and trypticase were not required for growth, whereas vitamins and trace element solutions were necessary for growth. The addition of 0.1–0.5% yeast extract stimulated the growth conditions.

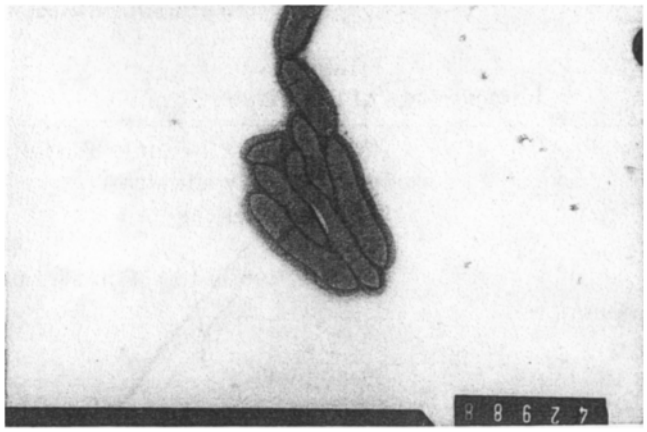

Fig. 1. Electron micrograph depicting the vegetative cells of strain RT9, ×6000.

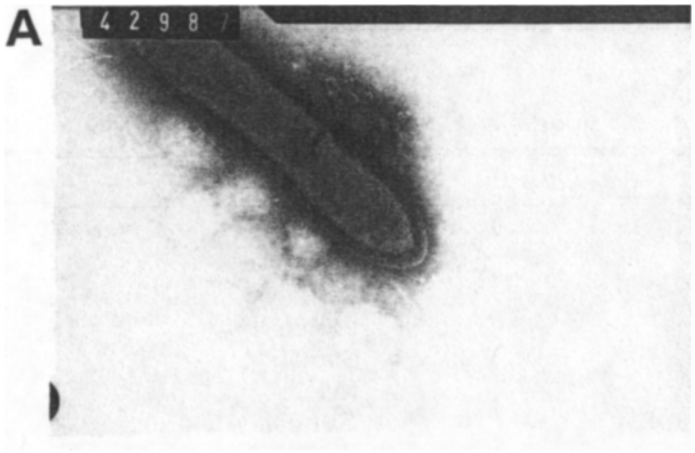

Fig. 2a. Negatively stained electron micrograph showing the location of flagella, ×15,000.

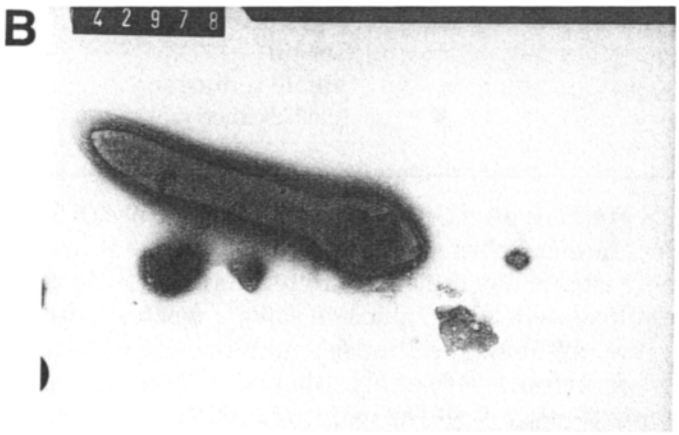

Fig. 2b. Negatively stained cells showing the presence of spores, ×13,000.

Table 1
Characteristics of *Clostridium* Strain RT9

Shape and size of cells	Rods 0.4–0.6×2–8 μm occurring singly or pairs
Flagella	Peritrichously situated
Spore	Oval to spherical
Gram stain	Negative
Colony	Circular, white to yellowish in roll tubes
Optimum temperature	35–37°C
Optimum pH	6.8–7.2
G+C content in DNA	39–40 mol%
Carbohydrates used	Glucose, cellobiose, and usable cellulose
Products formed	Ethanol, acetic acid, lactate, butyric acid, acetone, H_2 and CO_2
Reaction to oxygen	Strictly anaerobe

Table 2
Biochemical Characteristics of Strain RT9

Substrates	Growth	Substrates	Growth
Adonitol	+	Malonate	–
Arabinose	–	Melibiose	+
Argnine	–	Raffinose	+
Cellulose	+	Sorbitol	–
Cellobiose	+	Starch	Weakly
Dulcitol	+	Xylose	+
Esculin	+	Gumrabic	Weakly
Fructose	+	Catalase	–
Glucose	+	Indole-nitrate	–
Glycerol	+	Citrate	–
Galactose	+	Nitrate	–
Insitol	–	Voges-proskauer	–
Lysine	–	Gelatin	Not liquified
Mannose	+	Sulfate reduction	–
Maltose	–	Yeast extract	+
Mannitol	+		

The mol% guanine plus cytosine for strain was 39–40. Some morphological and biochemical characteristics of bacterium are listed in Tables 1 and 2. The new isolate has the ability to ferment the wide range of carbohydrates. Cellulose, cellobiose, glucose, salicin, fructose, dulcitol, esculin, mannitol, xylose, melibiose, raffinose, mannose, glycerol, adonitol, and galactose were fermented very easily. Malonate, lysine, arginine, sorbitol, inositol, arabinose, and maltose were not fermented. Gum arabic and starch were weakly hydrolyzed. The strain was catalase, indole, citrate, nitrate, and voges-proskauer negative. Gelatin was not liquified and did not reduce sulfate.

Table 3
Comparison of *Clostridium* Strain RT9 and *Cl. thermocellum* (JW_{20})

Substrate	Products, m*M*			
	Ethanol	Acetone	Acetate	Butyric acid
Cl. thermocellum (JW_{20})				
Cellobiose (1%)	7.68	0.60	3.99	0.77
CMC (1%)	2.41	0.28	4.95	0.35
Avicel (1%)	3.43	0.24	7.95	1.72
Clostridium strain RT9				
Cellobiose (1%)	23.79	0.038	11.14	6.9
CMC (1%)	1.58	0.24	6.2	0.62
Avicel (1%)	1.28	0.20	8.03	2.60

Table 4
Comparison of Product Formation of RT9 Strain with Other Mesophilic Cellulolytic Organisms for the Total Hydrolysis of 0.5% Filter Paper

Organisms	Products, m*M*			Filter paper hydrolysis, d
	Acetate	Ethanol	Butyrate	
Clostridium sp. RT9	10.0	12.5	3.7	13
Cl. papyrosolvens	1.4	0.7	0.2	28
Cl. cellulovorans	2.5	0.5	0.8	28
Cl. polysaccharolyticum	0.7	0.2	0.1	28
Bact. cellulosolvens	1.1	77.0	-	8

Major fermentation products of cellulose, cellobiose, and glucose were ethanol, acetate, lactate, butyrate, butanol, acetone, carbon dioxide, and hydrogen. Pyruvic acid and succinic acid were not detected.

Fermentation products from cellobiose, Avicel, and Carboxymethylcellulose (CMC) were compared with *Cl. thermocellum*, and are given in Table 3. Ethanol and acetate production from this strain was significantly higher than that of *Cl. thermocellum*. Comparison of filter paper hydrolysis with other mesophilic cellulytic bacteria is shown in Table 4. Complete hydrolysis of 0.5% filter paper occurred quickly as compared to other mesophilic cellulytic bacteria with the highest yield of ethanol, acetate, and butyrate.

On the basis of morphological, cultural, and physiological characteristics mentioned in group III of the classification of clostridia by Smith and Hobbs (*13*), strain RT9 belongs to the family Bacillaceae and the genus *Clostridia*. *Clostridium* strain RT9 differs uniquely from reported mesophilic *Clostridia* in morphological, fermentation, and biochemical characteristics.

Table 5
Cellulolytic Activities of *Clostridium* Strain RT9 After Growth in Sigmacell-100

Cellulolytic enzyme	Activity, μmol/mL/min
CMC-ase (endoglucanase)	0.22
β-glucosidase	0.35
β-xylosidase	0.16
Filter paper (endo- and exoglucanase)	2.0*
Xylanase	ND†

*μmol/mL/h.
†ND: Not determined.

Biochemical properties of the strain do not match with biochemical profiles mentioned in any of the known mesophilic clostridial species. Strains can ferment varieties of hexoses and pentoses, such as *Cl. polysaccharolyticum,* but differ from fermentation products and G+C contents (*7*).

Morphological and physiological characters are not a sufficient basis for the separation of bacterial species. According to the classification of genus *Bacilli* in Bergey's manual of systematic bacteriology, bacterial species can also be differentiated within the genus on the basis of DNA-DNA homology determinations (*13*). The mol% G+C content of this strain is 39–40, which is within the range of *Clostridia* species. The isolate can be readily distinguished from *Cl. chartatabidum, Cl. longisporum, Cl. papyrosolven, Cl. cellobioparum,* and *Cl. loch headii* on the basis of G+C homology determined by the thermal denaturation method. The mol% G+C ratio of this strain is near to the 41 mol% G+C content of *Cl. cellulolyticum,* but the major difference lies in biochemical tests and production of formate from cellulose by *Cl. cellulolyticum* (*14,15*).

Cellulytic Enzyme System

Clostridium strain RT9 produced complete enzyme complex when grown on cellulose in a batch culture. Endoglucanase, exoglucanase, β-glucosidase, and β-xylosidase activities were determined extracellularly in exponentially growing culture on cellulose (Table 5). Total β-glucosidase and endoglucanase activities were higher than that of β-xylosidase and exoglucanase. Xylanase activity was not detected. Cellulose binding activities, exo- and endoglucanase, were not determined. All activities were measured between pH 5–6 and optimum temperature 40–50°C, depending on the substrate used. Endo- and exoglucanase activities were not present when culture was grown on cellobiose. The multiple-enzyme system produced by this strain contained the necessary cellulase complex required for the conversion of cellulose to glucose. This involves endoglucamase (CMCase), exoglucanase (cellobiohydrolase), and β-1,4-gluco-

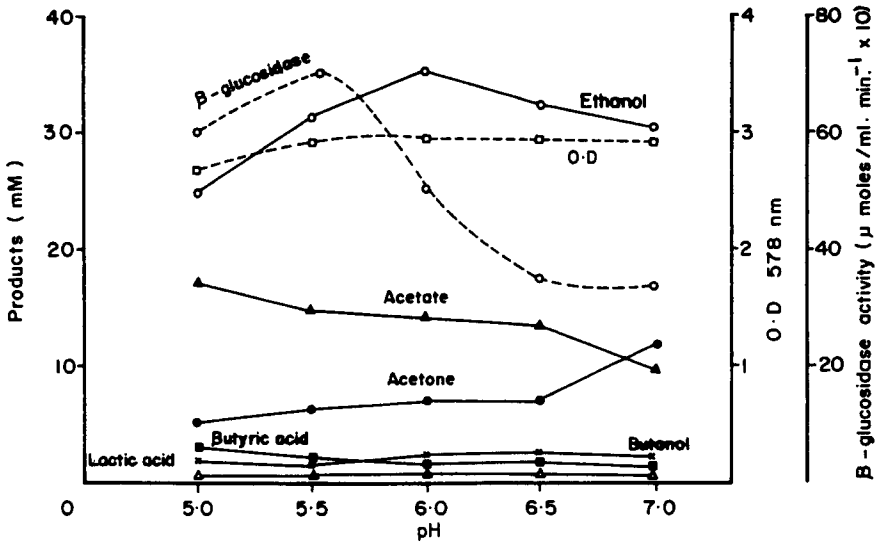

Fig. 3. Influence of pH on growth, products formation, and β-glucosidase production in a continuous culture of strain RT9 with 0.5% cellobiose in the feed at regulated dilution rate of 0.1 h^{-1}.

sidase (cellobiase), which act synergestically for the digestion of crystalline cellulose. According to some reports, endoglucanase randomly splits the cellulose polymer into smaller components, whereas exoglucanase removes one glucose unit at a time from the nonreducing end of the split molecule. β-1,4 glucosidase is the terminal enzyme. Cellobiose and cellodextrins are subsequently hydrolyzed to glucose by β-glucosidase, which may be the rate-limiting step in cellulose utilization. Until now, fungal *Trichoderma* and *Cl. thermocellum* cellulases were active and commercially important (2–3). The amount of cellulases produced by the strain RT9 was lower than that of *Trichoderma reesi* and *Cl. thermocellum*. However, activities are comparable to the cellulases of *Cl. acetobutylicum, Acetovibrio cellulolyticum,* and *Cl. cellulolyticum* (*16,17*).

Continuous Culture

Chemostat experiments were conducted under pH, dilution rate, and carbohydrate limitation to study the influence of these parameters on enzymes and product formation. Effect of pH was studied at a dilution rate of 0.10 h^{-1} with cellobiose (0.5%) as the limiting nutrient. Only β-glucosidase activity was produced in chemostat (Fig. 3). Endoglucanase, exoglucanase, and β-xylosidase activities were not expressed. Maximum β-glucosidase occurred at pH 5.5. This activity was 17-fold higher than the batch culture experiment. The ethanol and acetate production was also influenced by changing the pH. Maximum ethanol production was at pH 6.5, and acetate production was higher at acidic pH 5.0. When strain RT9 was grown in cellobiose-limited chemostat (0.5%) at different dilution rates and at regulated pH 5.6 (Fig. 4), maximal β-glucosidase was observed at a

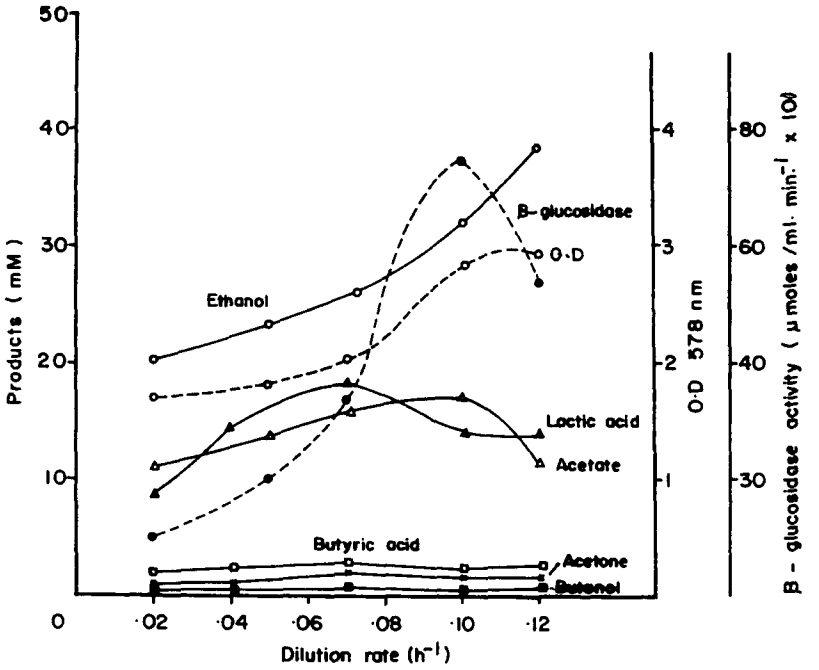

Fig. 4. Effect of dilution rate on growth, solvent concentration, and β-glucosidase production in a chemostat with 0.5% cellobiose at pH 5.6.

dilution rate of 0.10 h^{-1} and ethanol production at 0.10 h^{-1}. β-glucosidase activity declined at a lower and higher dilution rate than 0.012 h^{-1}. Ethanol production was twofold higher from each culture department. Production of lactic acid and acetic acid also increased with the change in dilution rate.

In carbon-limitation chemostat at pH 5.6 and dilution rate of 0.12 h^{-1}, the diauxic growth pattern of carbon utilization and β-glucosidase expression was observed. The highest level of β-glucosidase expression was observed when CMC was used as an inducer in the cellobiose-limited chemostat (Table 6). The expression of enzyme was decreased when culture was switched to carboxymethylcellulose (0.5%) or glucose (1%) as carbon source. Small amounts of reducing sugars were observed from chemostat experiments. CMC was not easily hydrolyzable at this dilution rate, and glucose was not a better inducer than cellobiose. The high concentration of glucose repressed the β-glucosidase activity produced by this strain. The maximum β-glucosidase activity was observed at a temperature of 45°C and a pH of 6.5. The activity was calculated on a percent relative basis (Fig. 5) because of continuous nutritional small changes in chemostat culture.

Some morphological changes were also observed in bacterium growing in chemostat. Cells were small, thick, and wide with swelled sporangium (Fig. 6) because of continuous nutritional changes in chemostat culture. The regulation of β-glucosidase has been studied previously in batch and continuous culture in *Bacterioides ruminicola* (*18*).

Table 6
Influence of Carbohydrates on Fermentation Products and β-Glucosidase Production*

Carbohydrates	OD, 570 nm	Reducing sugars, mg/mL	β-glucosidase μmol/mL/min	Ethanol m*M*	Acetone m*M*	Acetate	Butyrate m*M*	Lactate m*M*	
								D	L
Cellobiose 0.5%	3.0	0.15	7.5	40.0	0.06	12.6	0.04	5.2	10.0
Cellobiose 0.5% + CMC 0.05%	2.8	0.20	8.0	32.0	0.04	16.0	1.0	4.32	9.3
CMC 0.5%	1.5	0.12	2.3	14.0	0.20	10.6	2.0	2.1	5.0
Glucose 1%	5.7	N.D	2.7	60.15	0.10	24.40	N.D	5.6	12.0

*Products and enzyme activity were measured during growth in a chemostat at pH 5.6 and dilution rate of 0.10 h^{-1}.

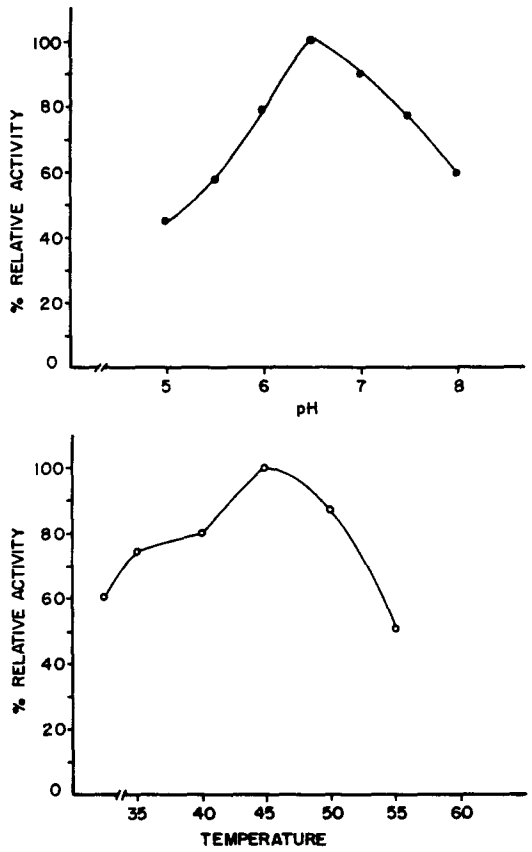

Fig. 5. Effect of pH and temperature on β-glucosidase activity.

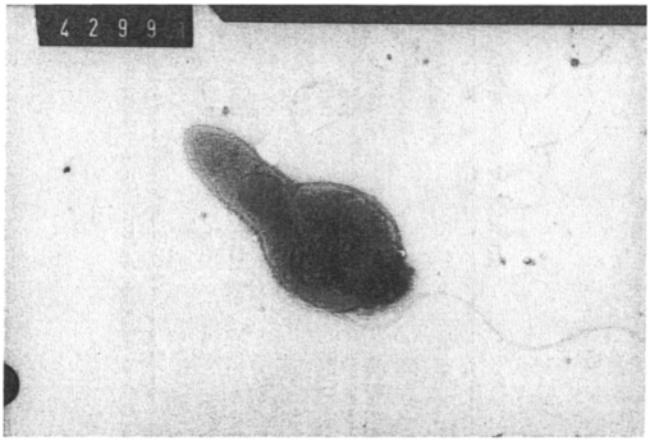

Fig. 6. Electron micrograph showing the morphological changes of strain RT9 in a chemostat ×15,000.

The results indicate that *Clostridium* strain RT9 is a new ruminal mesophilic cellulytic *Clostridia* that produces a complete enzyme system for conversion of cellulose into ethanol. It is also of interest that it produces considerable amounts of ethanol. The production of β-glucosidase and ethanol may prove useful in the development of technology for economically converting cellulose waste to chemical feed stocks.

ACKNOWLEDGMENT

This work was supported by Pakistan Atomic Energy Commision.

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