Thermoanaerobic Bacterial Fermentation for Production of Ethanol and Enzymes.

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Organismal Aspects of Thermophilic Anaerbic Fermentations

Chemical production by anaerobic bacterial fermentations was an important microbiological topic in the past due to both fundamental and applied aspects related to acetone-butanol production prior to its replacement by chemical synthetic routes from petroleum. Presently, the depletion and price-escalation of petroleum has regenerated a great interest in the potential of anaerobic bacteria to transform the renewable resouces such as biomass and wastes into chemical feed-stocks and fuels (4, 25, 26, 28, 30).

Thermoanaerobic fermentations are of interest to biotechnological development of novel processes for production of chemicals and fuels from biomass because of biological properties inherent to thermophilic anaerobic bacteria and process features associated with high temperature and anearobic condition. In general, thermoanaerobic ehtanol fermentation confers some beneficial aspects not common to the combined systems of enzymatic digestion of cellulosic biomass to glucose by fungal cellulase and ethanol production with yeast, because of direct conversion of polysaccharides itno ethanol, fermentation of both hexoses and pentoses, high metabolic rates, and facilitated end product rocovery (29, 30, 32).

A variety of thermophilic saccharolytic anaerobes have been siolated from self-heating (manure piles and wet soils) or volcaic features in nature (3, 17, 29, 23). These thermoanaerobic species display quite different properties in terms of growth characteristics, substrate utilization and end product formation.

Clostridium thermocellum which actively ferments cellulose and cellulose-hydrolysis products but not pentoses, sucrose or starch has been most widely investigated because it has an active, extracellular cellulase and it directly ferments cellulosic polymers to ethanol (14,32). Growth of this species is notably lower on cellulose (doubling time 7 hrs) than on cellobiose (2.4 hrs), suggesting that solubilization of the polymer is a rate-limiting metabolic step (29).

The described thermophilic anaerobes vary considerably in their metabolic, cellular and growth properties. *Clostridium thermohydrosulfuricum* utilizes a broad range of substrates as an energy source including pentoses, starch, galactose and cellobiose but does not ferment cellulose (31). This species displays a growth temperature optimum of 65°C, a doubling time of 75 minutes on glucose, high ethanol/lactate or acetate ratio (31) and its growth is inhibited by high partial pressures of hydrogen.

Thermoanaerobium brockil is an asporogenous, gram-positive rod that ferments starch, cellobiose, and a variety of monosaccharides. This species grows optimally at 65-70°C and displays a high lactate/ethanol or acetate ratio and a generation time of 1.5 hr on glucose (30,33).

Thermobacteroides acetoethylicus is a nonsporing, gramnegative rod and its growth is inhibited by tetracycline and penicillin G but not by hydrogen. It produces ethanol and acetate as main fermentation products but doesn't produce lactate (3). This species has an optimal growth temperature at 65°C, utilizes a variety of monosaccharides and displays a doubling time around 20 minutes.

Clostridium thermosulfurogenes is a gramnegative rod that has a double-layered wall with-

out an outer membraneus layer on electron micrographs (24). This notable species can utilize pectin as an energy source and transform thiosulfate to elemental sulfur which deposits in the culture medium and on cells. It also ferments a variety of carbohydrates such as xylose, galactose, glucose, mannose, cellobiose and sucrose, and displays a growth rate of about 2 hours on glucose or pectin. Ethanol, H_2/CO_2 acetate and lactate are formed as fermentation products of *C. thermosulfurogenes* on glucose.

Recently, applied research on thermophilic saccharide fermentations has focused on the process fundamentals and improvements associated with direct conversion of cellulosic substrates to ehanol. This was accomplished by the use of cocultures comprised of *C. thermocellum* and a second ethanol producing species (25, 26, 27). Coculture fermentations have been suggested to be of practical value by several investigators because of the improved ethanol yields from biomass. For example, when *C. thermocellum* was grown in coculture with *C. thermohydrosulfuricum* on cellulosic substrates, its ethanol yield drastically increased while acetate decreased and considerably more substrate was fermented (32).

The biochemical basis for different ratios of ethanol to total end products formed by different thermophilic species is related to subtle differences in the specific activities and regulatory properties of the enzymes which control cellular electron flow during fermentation (14, 30, 32). By adding modulators of specific enzyme activities to culture media, electron flow can be altered in species to drastically change the ratio of reduced end products formed or to produce a new product. For example, H_2 addition to *T. brockii* culture increased ethanol and lactate production, while acetone addition decreased H_2, lactate and ethanol but increased acetate and resulted in isopropanol production.

Although the biotechnological potentials of thermophilic saccharide fermentations have been recently emphasized because of the active, stable industrial enzymes in addition to chemical and fuel production, relatively little is known about both biochemistry and physiology of the key enzymatic steps responsible for biotransformation of saccharides.

Only a few enzymes isolated from thermoanaerobic bacteria have been purified and characterized. The extracellular cellulase complex of *C. thermocellum* was examined in great detail but only endoglucanase activity was purified (21). The intracellular enzymes, cellobiose phosphorylase (1,2) and cellodextrin phosphorylase which phosphorylate cellobiose and cellodextrins conserve the hydrolysis energy of beta-1,4 bond by reacting with inorganic phosphate. The higher cellular growth yield of *C. thermocellum* on cellobiose than on gucose was attributed to use of cellobiose phosphorylase (20).

The alcohol dehydrogenase of certain thermophilic anaerobes such as *T. brockii* and *C. thermohydrosulfuricum* is of practical interest for production of NADPH and chiral solvents, and analytical solvent detection due to its high thermostability, high solvent stability, broad substrate specificity and rare carbonyl si-face stereo specificity in hydrogen transfer (15,16). *C. thermosulfurogenes* forms a thermostable, extracellular pectate hydrolase during growth (22,24). However, regulation of enzyme production or the catalytic efficiency of the pectate hydrolase was not examined. Notably, it has been suggested that ethanol inhibits glycolytic enzymes of thermophilic anaerobes (5).

Physicochemical Properties of Amylolytic Enzymes from Thermoanaerobes.

Essentially, nothing is known about the biochemical attributes of thermophilic bacteria that actively ferment starch to ethanol at greater than 60°C. Nonetheless, thermoanaerobic bacteria can often possess faster metabolic rates and more thermostable enzymes than mesophilic microorganisms (22). *Clostridium thermosulfurogenes*, an anaerobic bacterium which ferments starch into ethanol at 62°C, produces an active extracellular beta-amylase, which hydrolyzes the alpha-1,4-glucosidic linkages in an exo-fashion from the non-reducing end of starch-type substrates and produces both maltose and beta-limit

dextrins (7). The species also produces intracellular glucoamylase but not pulllanase activity. *Clostridium thermohydrosulfuricum*, a thermophilic anaerobic bacterium produces cell-associated glucoamylase and pullulanase but not beta-amylase activity (8).

The beta-amylase, glucoamylase, and pullulanase which are widely used in the industrial biotransformation processes of starch into glucose, maltose, fructose or dextrins, are extremely thermoactive and thermostable, and have the pH optima in the acidic ranges (Table 1.)

Table 1. Physical Properties of Thermophilic *Clostridium* Amylase.

Enzyme Activity	Temperature		pH	
	Optimum	Stability	Optimum	Stability
beta-Amylase	75℃	Up to 80℃	5.5-6.0	3.5-6.5
Glucoamylase	75℃	Up to 75℃	4.0-6.0	4.5-6.0
Pullulanase	85℃	Up to 85℃	5.5-6.0	4.5-5.5

Generally, the food industries need the more thermostable glucoamylase, beta-amylase or pullulanase are conductd at higher than 60°C in order to prevent the microbial contamination. Also, amylases with pH optimum and pH stability in the acidic ranges are industrially favorable because the starch conversion is carried out in acidic pH to reduce the byproduct formation. Therefore, the amylases from *C. thermosulfurogenes* and *C. thermohydrosulfuricum* are outstanding in terms of thermostability and activity at high temperature to be used in the industrial starch transformation.

Enhancement of Amylases and Ethanol Production from Starch by Co-culture System.

An interest in thermophilic saccharide fermentations has been generated because of the production of industrial feedstock chemicals or fuels from biomass, and the potential development of more active and stable industrial enzymes (16, 25, 29, 34). Starch as a major component of agricultural crops is an important substrate for chemical and enzyme production because its chemical composition and higher density than other forms of biomass facilitates prolonged storage, and decreased transportation and pretreatment costs.

Thermoanaerobic bactria are suggested to have the applicational potential for production of active and thermostable amylases as well as for direct bioconversion of starch into ethanol (29). However, considerable improvements in thermophilic fermentation technology are needed to improve the yield of thermostable amylases or ethanol in order to demonstrate process feasibility. Therefore, co-culture fermentation system using two novel thermoanaerobes, *C. thermosul-*

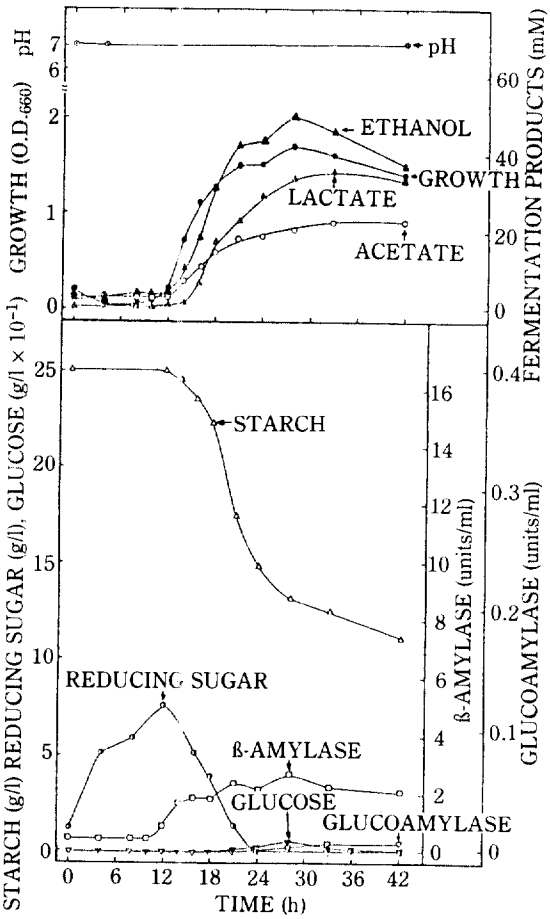

Fig. 1. Monoculture starch metabolism time course of *C. thermosulfurogenes* at pH 7.0 in a gassed fermentor. The experiment was conducted in a fermentor that contained 650 ml of modified TYE medium and 2.5% starch and which was gassed with N_2-CO_2 (95:5) and incubated at 60°C with mixing. The monoculture was initiated by injection of a 5% inoculum grown on the same medium.

furogenes and *C. thermohydrosulfuricum* was developed for the simultaneous and enhanced production of both amylolytic enzymes (i.e., beta-amylase, pullulanase and glucoamylase) and ethanol.

Fig. 1 shows the fermentation time course of *C. thermosulfurogenes* grown on 2.5% starch at pH 7.0. The mono-culture displays an initial lag phase where a high amount of reducing sugars accumulate, probably as a result of active, extracellular beta-amylase action (7). The accumulated reducing sugars are rapidly consumed when growth initiated; and, both beta-amylase production and end product formation occurs simultaneously during the logarithmic growth phase. Starch is incompletely used, however, which implies that the beta-limit dextrins formed by beta-amylase are not further metabolized due to lack of pullulanase, a starch-debranching enzyme.

However, the mono-culture of *C. thermohydrosulfuricum* displays an unusual fermentation pattern as illustrated in a starch metabolism time course of the species grown under conditions of

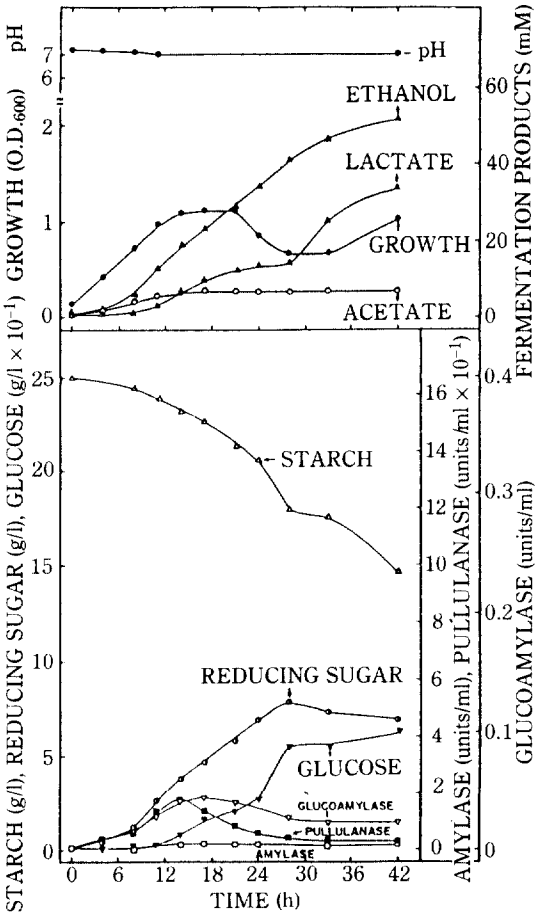

Fig. 2. Monoculture starch metabolism time course of *C. thermohydrosulfuricum* at pH 7.0 in a gassed fermentor. The experimental conditions were the same as in the legend to Fig. 3, except for inoculum source, and incubation was carried out at 65°C.

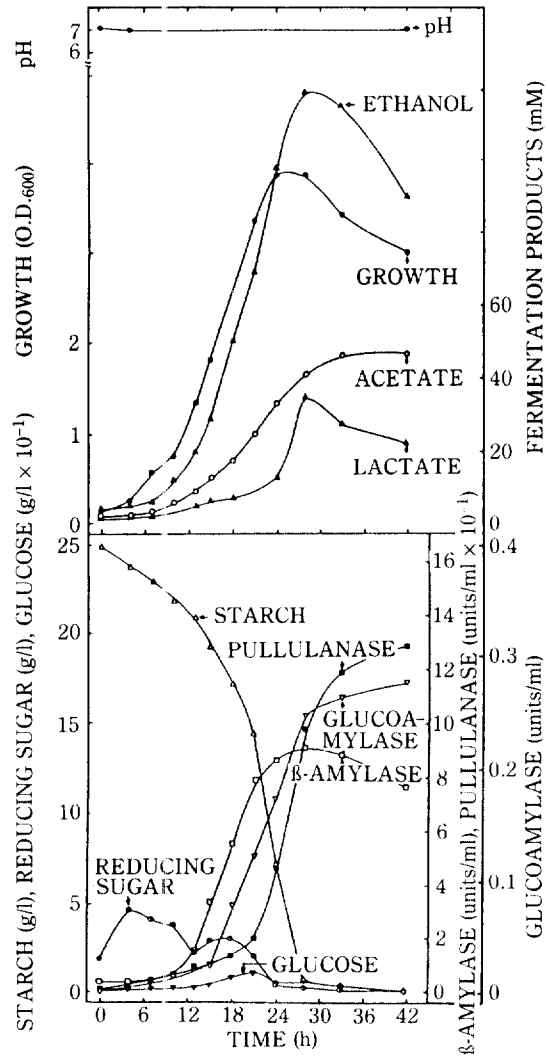

Fig. 3. Coculture starch metabolism time course of *C. thermosulfurogenes* and *C. thermohydrosulfuricum* at pH 7.0 in a gassed fermentor. Cocultures were initiated by injection of a 2.5% inoculum of each organism. Other conditions were the same as in the legend to Fig. 3.

continuous gassing and pH control (Fig. 2). The unusual growth pattern indicates extensive cell lysis, probably due to the low amylolytic activity (8). Starch also is not completely utilized by this species under the growth conditions employed. Interestingly, glucose accumulates continuously during the entire culture period, which may cause the catabolite repression for amylase synthesis.

The dramatic improvement in starch metabolism observed when co-cultures are grown at pH 7.0 under conditions of continuous gassing is shown in Fig. 3. Starch is rapidly degraded and completely consumed during the fermentation time course. Co-culture fermentations consume starch completely and more rapidly than monocultures under similar conditions (see Fig. 1 and Fig. 2). Most importantly, the co-culture produces more cells, total amylases and ethanol. Balanced growth is achieved by both species in this coculture as evidenced by the pattern of growth, complete starch consumption, the simultaneous production of three amylases, and the ratio of end product formation. The most remarkable feature of the co-culture fermentation is an enhanced rate and yield of ethanol production and the significant enhancement of glucoamylase and pullulanase production. The reason of enhanced amylase production may be that growth of both species competitively removes low molecular substrates (e.g., glucose) liberated by the coordinate action of amylases, resulting in alleviation of catabolite repression of amylase synthesis. Therefore, simultaneous and enhanced production of the thermostable amylases and ethanol by co-culture starch fermentations may enable an inexpensive method for producing ethanol and industrial amylases needed in both starch saccharification and ethanol production processes (9).

Strain Improvement for Enhanced Production of Ethanol and Amylases

In order to enhance amylolytic enzyme production as well as ethanol production from thermoanaerobic fermentations, regulatory mutants, i.e., catabolite repression-resistant mutants and

Table 2. Comparison of Growth, beat-Amylase and Fermentation Product Formation by wild Type and Mutant Strains of *C thermosulfurogenes*[a].

Strain	Growth Substrate[c]	Growth ($O.D_{660}$)	μ_{max} (hr^{-1})	β-Amylase (U/ml)	substrate consumed (μmol glucose)	End Product (μmol/tube) Ethanol	Acetate	Lactate	H_2	CO_2
Wild Type	Glucose	1.20	0.05	0.2	278	245	136	180	288	381
	G + S	1.30	–	1.6	–	–	–	–	–	–
	Starch	0.93	0.41	6.0	185	246	116	7	270	362
CRR[b] Mutant	Glucose	1.20	0.47	0.1	278	154	111	74	248	565
H 35	G + S	1.30	–	10.8	–	–	–	–	–	–
	Starch	1.00	0.40	16.2	203	268	104	4	270	372
CCRR[b] Mutant	Glucose	1.10	0.58	18.0	278	136	143	33	337	579
H 227-7	G + S	1.15	–	18.5	–	–	–	–	–	–
	Starch	1.05	0.52	20.7	198	244	114	0	275	358
Hyperproductive	Glucose	1.35	0.52	16.0	278	120	138	10	365	558
Mutant	G + S	1.40	–	34.6	–	–	–	–	–	–
	Starch	1.05	0.52	46.3	187	299	110	0	243	399

a. Cells were cultivated in a pressure tube comtaining 10 ml of TYE medium plus each substrate at 60 ℃ for 20 hrs.
b. CRR and CCRR abbreviate Catabolite Repression-Resistant, and Constitutive and Catabolite Repression-Resistant, respectively.
c. 0.5% Glucose or starch was used, and G+S means of 1.5% glucose and 0.5% starch.

constitutive mutants, of *C. thermousulfurogenes* (11) and *C. thermohydrosulfuricum* (12) were isolated using 2-deoxyglucose as a non-metabolizable catabolite repressor. The beta-amylase activities, growth properties and fermentation balances of *C. thermosulfurogenes* wild type and mutant strains grown on various carbon sources are compared in Table 2. The catabolite repression-resistant mutant H35 produces about 3-fold more beta-amylase on starch medium than the wild type; and, upon the addition of glucose to starch medium, it still produces high activity of beta-amylase but not on glucose medium. In contrast, H227-7 and H12-1, constitutive mutants produce 3 fold and 8 fold more beta-amylase activities, respectively than the wild type on starch medium. In addition, these regulatory mutants produce moree thanol than the wild type as a result of switching from lactate to ethanol production. The constitutive mutants H227-7 and H12-1 also grow more rapidly on starch medium than the wild type.

The catabolite repression-resistant mutants of *C. thermohydrosulfuricum* also produce more glucoamylase and pullulanase activities than the wild type on starch medium (Table 3). The regulatory mutants also grow more rapidly and produce more ethanol on starch medium than the wild type. Therefore, achievement of strain improvements with amylase mutants in *C. thermosulfurogenes* and *C. thermohydrosulfuricum* greatly advances the practical potential of using thermoanaerobic fermentations for industrial production of amylases as well as ethanol from starch. In addition, ethanol-tolerant mutants were isolated in *C. thermohydrosulfuricum* (18) and *C. thermocellum* (6) that can grow at high end product concentrations (i.e., ethanol., H_2). Therefore, selection of high ethanol tolerant and yield mutants from the regulatory mutants of *C. thermohydrosulfuricum* and *C. thermosulfurogenes* will be a future target for practical improvements because these mutants may result in achievement of enhanced enzyme and ethanol yields as a consequence of increased growth yield.

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Table 3. Comparision of Growth, Amylase Activities and Fermentation Product Formation by Wild Type and Mutant Strains of *C. thermohydrosulfuricum*[a].

Strain	Growth Substrate	Growth ($O.D_{660}$)	μ_{max} (hr^{-1})	Gluco-amylase (U/ml)	Pullula-nase (u/ml)	Substrate Consumed (μmol glucose)	End product (μmol/tube) Ethanol	Acetate	Lactate	H_2	CO_2
Wild Type	Glucose	0.97	0.36	0.000	0.00	278	498	50	83	48	548
	G + S	1.40	–	0.000	0.00	–	–	–	–	–	–
	Starch	0.80	0.17	0.030	0.23	270	461	26	54	39	487
CRR[b] Mutant	Glucose	0.39	0.36	0.000	0.00	139	306	43	48	28	349
267-143	G + S	1.35	–	0.020	0.23	–	–	–	–	–	–
	Starch	1.00	0.17	0.030	0.23	248	390	35	25	40	426
Hyperproductive	Glucose	1.40	0.37	0.000	0.00	278	551	27	47	34	578
Mutant Z21-109	G + S	1.30	–	0.026	0.24	–	–	–	–	–	–
	Starch	1.18	0.28	0.048	0.43	271	472	39	36	44	511

a. Cells were cultivated in a pressure tube containing 10 ml of TYE medium plus each substrate at 60 ℃ for 24 hrs.
b. CRR abbreviate Catabolite Repression Resistant.
c. 0.5% Glucose or starch was used as a carbon source, and G+S means the mixture of 1.5% glucose and 0.5% starch was used.

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