

Screening and Characterization of Lactate Dehydrogenase-producing Microorganism

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ABSTRACT : The objective of this work was to isolate a microorganism, able to produce high lactate dehydrogenase (LDH) activity, for use as a microbial feed additive. The LDH is an important enzyme for lactate conversion in the rumen, thereby possibly overcoming lactic acidosis owing to sudden increases of cereal in the diets of ruminants. In the present study, various bacterial strains were screened from a variety of environments. Among the isolated microorganisms, strain FFy 111-1 isolated from a Korean traditional fermented vegetable food called *Kimchi* showed the highest enzyme activity, along with retaining strong enzyme activity even in rumen fluid *in vitro*. Based on morphological and biochemical characteristics as well as compositions of cellular fatty acids plus API analyses, this strain was identified as *Lactobacillus* sp. The optimum temperature and pH for growth were found to be 30°C and pH 6.5, respectively. A maximum cell growth of 2.2 at A₆₅₀ together with LDH activity of 2.08 U per mL was achieved after 24 h of incubation. Initial characterization of FFy 111-1 suggested that it could be a potential candidate for use as a direct-fed microbial in the ruminant animals. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 10 : 1411-1416)

Key Words : *Lactobacillus*, Lactate Dehydrogenase, Direct-fed Microbials

INTRODUCTION

The rumen is a complex anaerobic microbial ecosystem, with many microbes and reactions. Although rumen microorganisms facilitate fiber digestion, domestic ruminants are often fed diets with high levels of energy source for promoting rapid growth or maintaining high levels of milk yield and meat production. Ingestion of large amounts of cereal concentrates provides carbon sources for rapid proliferation of rumen bacteria, such as *Streptococcus bovis* and *Lactobacillus* sp., which produce large amounts of lactic acid (Russell and Hino, 1985). An accumulation of lactate in the rumen results in a decline of pH in the rumen and, therefore, decreases in fiber digestion, dysfunction of the rumen, and in some case infectious diseases (Slyter, 1976; Russell and Rychlik, 2001) in ruminants may occur.

Direct-fed microbials have been studied as feed additives for improvement of health and production of livestock (Martin and Nisbet, 1992; Bae et al., 2003; Lee et al., 2003). Rumen manipulations with microbial additives have been attempted to reduce lactate accumulation in

ruminants fed high density diets (Aslan et al., 1995; Chaucheyras et al., 1996). Kung and Hession (1995) previously reported the possibility of inoculating ruminal fermentations with lactate-utilizing organisms such as *Megasphaera elsdenii* to overcome an accumulation of lactate. Although these attempts may improve the rumen function to some extent, there is a room for further improvement by exploiting lactate dehydrogenases (LDHs) responsible for a reversible reaction between lactate and pyruvate in rumen bacterial metabolism.

Baldwin and Milligan (1964) firstly reported the purification of pyridine nucleotide independent LDH from the anaerobic rumen bacterium *M. elsdenii* (formerly known as *Peptostreptococcus elsdenii*). Generally, microbial LDHs (Garvie, 1980) have two types of LDHs which are classified into nLDH (NAD linked) and iLDH (NAD independent). These LDHs have been identified in some microorganisms including *Lactobacillus* sp., *Bacillus* sp., *Saccharomyces cerevisiae*, and *E. coli* as well as rumen bacteria such as *M. elsdenii*, *Selenomonas ruminantium* and *Streptococcus bovis* (Scheifinger et al., 1975; Garvie, 1980; Hino and Kuroda, 1993). Some rumen bacteria are associated with lactate metabolism and have different LDHs (Hino and Kuroda, 1993; Gilmour et al., 1994). It was evident that their LDHs play an important role in the balance of lactate, which is the main culprit in the low ruminal pH (<6.0) and dysfunction of the rumen.

The objectives of this study were to isolate an LDH-producing microorganism able to be activated in fluid of rumen acidosis practically, and to investigate culture conditions for its optimal growth together with its high LDH activity in view of an application as a microbial supplement in ruminants.

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MATERIALS AND METHODS

Experiment 1

Screening of LDH-producing strains : Three hundred and twenty microorganisms isolated from various sources such as foods, plant roots, rumen contents, animal feces, and soils in Korea were tested for their abilities to produce acids. The isolates were spread onto acid detection agar medium (TSY) containing 17 g of tryptone, 3 g of soytone, 6 g of yeast extract, 2.5 g of glucose, 5 g of NaCl, 0.5 g of KH_2PO_4 , and 0.5 g of K_2HPO_4 in 1 L of distilled water (pH 6.5), together with adding 0.4 g of bromocresol green, and then incubated for 16 h at 39°C. Yellow colonies were selected and were grown separately in a basal medium by incubation at 35°C. The composition of a basal medium (g/L) was yeast extract (3), malt extract (3), glucose (10) and peptone (5). The selected isolates were used to screen LDH-producing strains by confirming zymogram analysis in cell extracts separated by non-denaturing PAGE (see section for zymogram analysis). In addition, to compare LDH activities under acidic *in vitro* rumen condition, the cell-free extract (0.5 mg protein per 0.2 mL) was treated with the acidic rumen fluid (1.8 mL). The acidic rumen fluid was obtained from *in vitro* rumen fermentation by using a method as reported previously (Kung and Hession, 1995). After enzyme reaction at 37°C for 20 min, the remaining lactate in the reaction mixture was measured by HPLC (Varian 9012, Optimize Technologies Inc., USA), equipped with an UV detector, using a carboxylic acid column (25 cm×4.6 mm, Supelco, USA). Two hundred mM of phosphoric acid was used as the mobile phase at a flow rate of 0.8 mL/min.

Preparation of cell-free extract and enzyme assay : Cells were harvested after 20 h of incubation and centrifuged at 10,000 g at 4°C for 10 min. The bacterial pellets were washed twice in 50 mM Tris-HCl buffer (pH 7.0) and centrifuged again. Cells were resuspended in Tris-HCl buffer and then sonicated (VCX 400, Sonic & Materials Inc., USA) at 4°C for 10 min (pulse on 3 s, pulse off 5 s, 20 kHz and pulse 40%). It was found by means of a light microscope (Olympus ABX-50, Japan) that approximately 80% of cells were disrupted by sonication. After removing the cell debris by centrifugation (12,000 g, 10 min), the supernatant was used as the cell-free extracts. The protein concentration was determined by the method as reported previously (Bradford, 1976). The LDH activity was measured using the same method as reported previously (Bergmeyer, 1974). An assay solution (1 mL) consisted of 50 mM Tris-HCl (pH 6.5), 12.5 mM DL-lactate (sodium salt), and 1.5 mM NAD. Depending on the activity of extracts, 50-100 mg of protein was added to the reaction assay solution and then the enzyme reaction was carried out at 37°C for 5 min. The enzyme reaction was stopped by

heating and the reduction of NAD resulting from the enzyme reaction was determined by measuring ΔA_{340} (Specord S100, Analytik Jena, Carlzeiss Technology, Germany) per min in a range of between 0.005 and 0.05. One unit (1 U) of LDH activity was defined as the reduction of 1 μmol of NAD per min.

Zymogram analysis for LDH activity in cell-free extracts : The cell-free extracts (10 μL) were applied on native non-denaturing gels. For the native non-denaturing PAGE, both SDS and 2-mercaptoethanol were omitted from the 10% gel (Laemmli, 1970) and protein samples were not heated before loading (Garfin, 1990). For staining the LDH, the gel after electrophoresis was equilibrated in 50 mM Tris-HCl buffer (pH 6.5) at 4°C for 10 min and then incubated at 37°C for 30 min with a substrate solution of 2 mM DL-lactate. Thereafter, the gel was transferred to the LDH staining solution (Sigma kit 705 A) and was incubated until dark-brown bands developed. For comparative purposes, two commercial enzymes (Sigma L 5275, 15,000 U and Sigma L 2011, 2,500 U) were also used as indicators of LDH activation.

Experiment 2

Identification and characterization of isolated strain: In order to identify the isolated strain, morphological and physiological characteristics were examined according to the methods described previously (Collins and Lyne, 1976; Gerhardt et al., 1981; Peter et al., 1986). Carbon utilization, optimum pH and temperature for growth, hemolysis test, homo-hetero fermentation test, and Gram staining were also investigated. To determine the fatty acids composition of the strain, twenty mg of the dried cells at 105°C for 3 h was directly methyl-esterified with 7% HCl in methanol (Hirano et al., 1990). Fatty acids composition of isolated strain was then measured by gas chromatography (Hewlett Packard HP 6890, USA), equipped with a capillary column (HP-Innowax, USA) under the column temperature of 210°C. Fatty acid methyl ester (FAME) analyses were carried out by using the Microbial Identification System (MIS, Microbial ID Inc., Newark, DE). Identification of the FAME and subsequent data management were done as the method described previously by Moore et al. (1994). Also the isolate was tested for its sugar utilization by using an API kit 50 CHL (bioMerieux, Inc., France) together with the API identification software and analytical profile index (Fleet et al., 1984).

Experiment 3

Optimization of growth medium for obtaining high LDH activity : The composition of a fermentation medium per L for cultivation of the isolated strains was as follows: 10 g of peptone, 1 g of yeast extract, 10 g of glucose and 1.0 g of KH_2PO_4 . Unless otherwise specified, the medium was

Table 1. Isolated strains and their lactate dehydrogenase (LDH) activities in bacterial cell-free extracts

Strain	Origin	Protein (mg/mL)	LDH activity (U/mg)
C 21-1	Rumen fluid	5.88±1.20	0.26±0.05
W 24-2	Soil	6.10±0.70	0.01±0.00
FF 29-3	Soybean paste	4.97±0.90	0.02±0.01
F 48	Animal feces	1.53±0.45	0.35±0.11
V 58-1	Green bean spout	6.74±0.70	0.03±0.02
V 96-1	Grape	3.38±1.20	0.12±0.06
S 108	Soil	1.77±0.36	0.06±0.03
Ry 4	Garlic	4.81±0.22	0.03±0.01
Iy 12-2	Soil	5.81±0.31	0.02±0.01
Fy 110-4	Animal feces	10.46±1.34	0.01±0.00
Fy 110-8	Animal feces	2.75±0.35	0.27±0.05
FFy 111-1	<i>Kimchi</i>	1.80±0.16	0.57±0.04

adjusted to pH 6.5 before sterilization. In order to optimize the medium for a selected strain (FFy 111-1), various carbon sources (glucose, fructose, galactose, maltose, mannose, lactose, cellobiose, sucrose, soluble starch, sodium acetate, pyruvic acid, butyric acid, salicine, D-lactate and L-lactate), nitrogen sources (NH_4Cl , NH_4NO_3 , NaNO_3 , $(\text{NH}_4)_2\text{HPO}_4$, $(\text{NH}_4)_2\text{SO}_4$ and KNO_3), and minerals sources (NaCl , CoSO_4 , KH_2PO_4 , MgSO_4 , CaCl_2 , KCl , BaCl_2 , CuSO_4 and MnSO_4) were tested. To choose best carbon, nitrogen, and minerals, only the corresponding component (glucose, peptone, and KH_2PO_4) was replaced in the fermentation medium. Forty-mL test tubes containing 10 mL of the fermentation medium were employed and all incubations were performed in triplicate.

Optimization of environmental conditions: Temperature optima for growth of strain FFy 111-1 together with its LDH activity using an optimized medium was examined at different temperatures ranging from 20 to 50°C. pH optima was also studied at different pH values ranging from 5.0 to 7.0. Unless otherwise specified, shaking incubation was carried out at 35°C for 20 h. Bacterial growth was determined by measuring the increase in absorbance at 650 nm in a spectrophotometer (Specord S100, Analytik Jena, Carlzeiss Technology, Germany).

Time-course batch growth under optimal conditions : A typical batch culture kinetics of strain FFy 111-1 on 2% fructose medium containing 4% yeast extract and 0.05% KH_2PO_4 at 30°C and pH 6.5 was carried out for 32 h to investigate maximum growth and LDH activity.

RESULTS AND DISCUSSION

Experiment 1

Isolation of LDH-producing bacteria: Initially, screening of LDH-producing bacteria was carried out based on yellow zone appearance around their colonies on acid detection agar plates since acids are the major end products

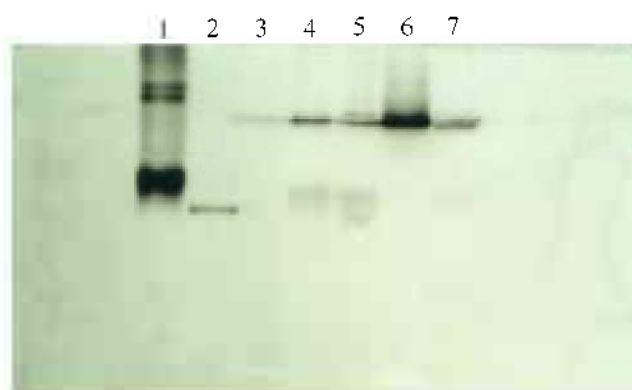


Figure 1. Zymogram for lactate dehydrogenase (LDH) activity in cell extracts separated by native non-denaturing polyacrylamide gel electrophoresis. Lane 1: L-nLDH, Sigma L 5275; Lane 2: D-nLDH, Sigma L 2011; Lane 3: Strain S 108; Lane 4: Strain Fy 110-8; Lane 5: Strain C 21-1; Lane 6: Strain FFy 111-1; Lane 7: Strain F 48.

(pyruvate or lactate) by LDH. Among the 320 isolates, 184 strains gave yellow zones around their colonies. Some of the primarily isolated strains were tested to compare their abilities for LDH production. There was considerable variation between the different strains, and data for strains are shown in Table 1. Strain C 21-1, F 48, Fy 110-8, and FFy 111-1 showed relatively high levels of LDH activity. The levels of LDH activity were also confirmed by zymogram analysis as shown in Figure 1. Accordingly, it was possible to select better LDH-producing bacteria such as Fy 110-8 and FFy 111-1 based on the levels of dark-brown bands developed by LDH reaction in staining solution.

Another consideration of strain selection for a ruminant feed additive is its activity and stability under acidic rumen fluid conditions. Therefore, various strains were tested to compare their enzyme activity in the presence of acidic rumen fluid (46 mM of lactate concentration) *in vitro*. When strains were tested in the acidic rumen fluid, the relative lactate concentrations with strain C 21-1, Fy 110-8, F 48 and FFy 111-1 were decreased to 91%, 89%, 84% and 78%, respectively (results not shown). Thus, strain FFy 111-1 was considered as the most suitable candidate strain for use as a microbial feed additive.

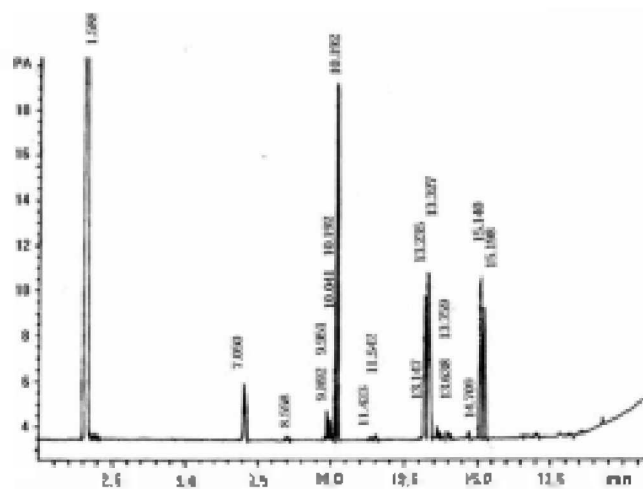
Experiment 2

Identification and characterization of strain FFy 111-1 : The morphological and physiological characteristics of strain FFy 111-1 were investigated to identify it in detail. An examination of colonies on TSY agar medium after incubation at 39°C for 16 h showed a yellow-white appearance. Strain FFy 111-1 was a rod-shaped, gram-positive, non-motile, and non-spore forming bacterium. It grew well between 25°C and 45°C, with an optimum pH in a range of 6.0 to 6.5. Strain FFy 111-1 showed negative

Table 2. Sugar utilization characteristics of isolated strain FFy111-1

Compound	Growth	Compound	Growth
Control	-	Esculin	+
Glycerol	-	Salicin	-
Erythritol	-	Cellobiose	-
D-Arabinose	-	Maltose	+
L-Arabinose	+	Lactose	-
Ribose	+	Melibiose	+
D-Xylose	+	Sucrose	-
L-Xylose	-	Trehalose	-
Adonitol	-	Inulin	-
β -Methyl-D-Xyloside	+	Melezitose	-
Galactose	+	Raffinose	-
Glucose	+	Starch	-
Fructose	+	Glycogen	-
Mannose	-	Xylitol	-
Sorbose	-	Gentiobiose	-
Rhamnose	-	D-Turanose	-
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	-
Mannitol	-	D-Fucose	-
Sorbitol	-	L-Fucose	-
α -Methyl-D-Xyloside	-	D-Arabitol	-
α -Methyl-D-Glucoside	+	L-Arabitol	-
N-Acetyl-Glucosamine	-	Gluconate	-
Amygdalin	-	2-Keto-Gluconate	-
Arbutin	-	5-Keto-Gluconate	-

+: utilized, -: not-utilized.

**Figure 2.** GC chromatogram of methyl-esterified fatty acids obtained from strain FFy 111-1. C14:0 (7.1 min), C16:0 (10.2 min), and C18:1 (13.3 min).

reaction in catalase, Voges-Proskauer, oxidase, urase, nitrate reduction, and gelatin liquefaction tests. As shown in Table 2, strain FFy 111-1 was able to ferment L-arabinose, ribose, D-xylose, β -methyl-D-xyloside, galactose, glucose, fructose, α -methyl-D-glucoside, esculin, maltose and melibiose. Therefore, it was considered to belong to *Lactobacillus* species. In addition, we performed fatty acid methyl ester

Table 3. Effects of fructose, yeast extract and phosphate on growth and lactate dehydrogenase activity of strain FFy 111-1 at 35°C and pH 6.5

Component	Concentration (%)	Growth (A_{650})	Total enzyme activity (U/mL)	Specific enzyme activity (U/mg protein)
Fructose	0.00	0.34±0.00	0.06±0.02	0.07±0.01
	1.00	0.82±0.09	0.23±0.05	0.13±0.03
	2.00	0.87±0.03	0.28±0.04	0.16±0.02
	3.00	0.78±0.02	0.27±0.05	0.13±0.02
	4.00	0.69±0.03	0.26±0.06	0.12±0.03
	5.00	0.60±0.01	0.16±0.02	0.11±0.01
Yeast extract	0.00	0.31±0.01	0.06±0.01	0.07±0.01
	1.00	0.73±0.05	0.31±0.05	0.29±0.03
	2.00	1.04±0.07	1.19±0.13	0.87±0.08
	3.00	1.29±0.03	1.53±0.09	0.61±0.05
	4.00	1.49±0.04	1.69±0.07	0.30±0.04
	5.00	1.53±0.02	1.67±0.11	0.27±0.04
KH ₂ PO ₄	0.00	0.65±0.01	0.23±0.02	0.23±0.02
	0.01	0.65±0.06	0.23±0.05	0.23±0.04
	0.05	0.71±0.04	0.25±0.03	0.24±0.02
	0.10	0.50±0.01	0.20±0.02	0.19±0.02
	0.50	0.46±0.05	0.19±0.04	0.17±0.03
	1.00	0.44±0.01	0.18±0.02	0.17±0.01

(FAME) analyses as shown in Figure 2. The major FAMES were found to be long-chain fatty acids such as C14:0 (4.37%), C16:0 (31.55%), and C18:1 (29.62%). Thus, it is concluded that strain FFy 111-1 was related to *Lactobacillus brevis* as the most neighboring species with 98% similarity. It has been reported that *Lactobacillus* sp. is one of the dominant microflora with *Kimchi* (Yun and Kim, 1998; Hong et al., 1999; Kim et al., 2004).

Experiment 3

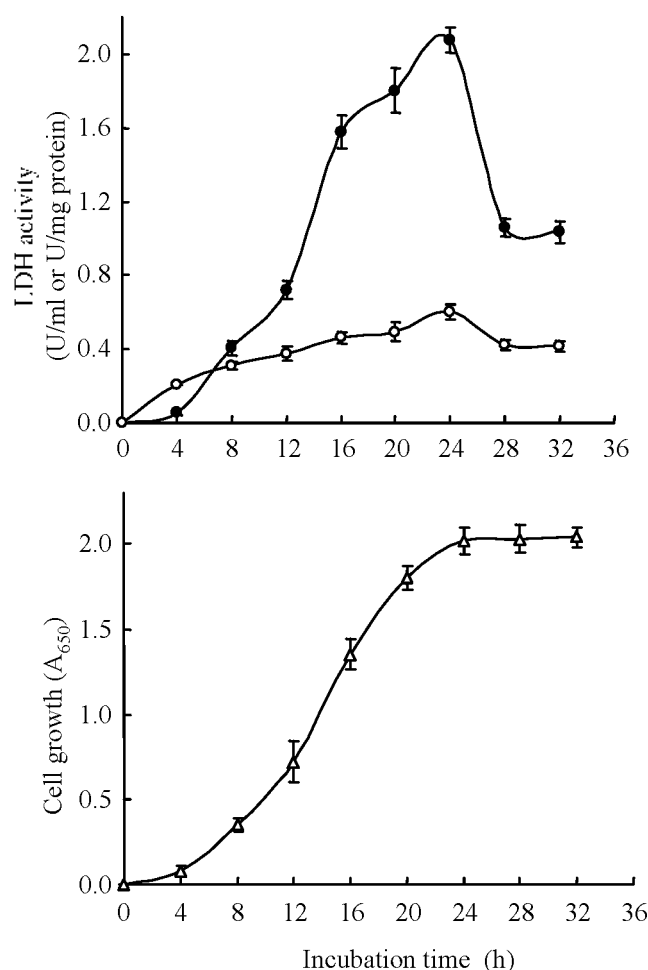
Effects of fructose, yeast extract, and potassium phosphate on cell growth and LDH activity: When strain FFy 111-1 was cultured with various carbon, nitrogen, and mineral sources, it was found that fructose, yeast extract, and potassium phosphate showed relatively higher LDH activities with good growth (data not shown). Therefore, the effects of fructose concentration, yeast extract concentration, and phosphate concentration on cell growth and LDH activity were studied in detail. Firstly, the effect of fructose was studied in a range of 0 to 5%. The total enzyme activity had optima between 2% and 4%, while an optimum fructose concentration for growth was 2% (Table 3). The cell growth gradually increased when a fructose concentration was increased up to 2%, beyond which there was a decline in growth probably due to the substrate inhibition caused by a high concentration of fructose. In contrast, the effect of fructose concentration on enzyme activity indicated a broad range with an optimum of 2%.

Table 4. Effects of pH and temperature on growth and lactate dehydrogenase activity of strain FFy 111-1

Item	Condition	Growth (A_{650})	Total enzyme activity (U/mL)	Specific enzyme activity (U/mg protein)
Temperature (°C)	20	0.62±0.01	1.24±0.05	0.66±0.03
	25	1.41±0.10	1.73±0.11	0.40±0.08
	30	1.90±0.06	1.84±0.07	0.40±0.04
	35	1.87±0.11	1.10±0.09	0.25±0.05
	40	1.46±0.04	0.68±0.06	0.27±0.04
	45	0.24±0.04	0.20±0.03	0.68±0.03
	50	0.13±0.00	0.11±0.02	0.93±0.04
pH	5.0	0.53±0.17	0.90±0.07	1.70±0.05
	5.5	0.65±0.02	1.02±0.05	0.87±0.08
	6.0	0.70±0.05	1.20±0.08	0.51±0.07
	6.5	0.71±0.01	1.31±0.04	0.49±0.05
	7.0	0.71±0.02	1.20±0.06	0.50±0.04

Secondly, varying initial concentrations of yeast extract in a range of 0 to 4% on growth and LDH activity, showed gradual increases in growth and total enzyme activity as summarized in Table 3. However, a maximum specific enzyme activity was obtained with 2% yeast extract. This means that the level of intracellular LDH activity was relatively higher when strain FFy 111-1 was grown with an optimal yeast extract concentration. Finally, the effect of potassium phosphate on cell growth and LDH activity was studied. Unlike the effects of fructose and yeast extract, a broad optimum growth together with enzyme activity was achieved within a concentration range of 0 to 0.05% with a maximum value at 0.05%, above which both growth and enzyme activity dramatically decreased as concentrations of potassium phosphate increased. Therefore when medium compositions for growth and enzyme production were all considered, optimum concentrations of fructose, yeast extract, and potassium phosphate were found to be 2%, 4%, and 0.05%, respectively.

Effects of pH and temperature on cell growth and LDH activity : The effect of pH on growth and enzyme activity was studied in the pH range of 5.0 to 7.0 as shown in Table 4. Increase in the pH from 5.0 to 6.5 resulted in the increased growth and the total enzyme activity. Although the growth was not changed above pH 6.5, however, the total enzyme activity decreased to some extent. It is interesting to note that a maximum specific enzyme activity occurred at pH 5.0, beyond which the activity decreased dramatically. Table 4 also shows the effect of temperature on growth and enzyme activity in the temperature range of 20°C to 50°C. The growth of FFy 111-1 was found to increase steeply when incubation temperature increased from 20°C to 30°C and remained constant until 35°C before decreasing rapidly. The optimum temperature for growth

**Figure 3.** Time-course batch culture of strain FFy 111-1 at 30°C and pH 6.5. Total lactate dehydrogenase (LDH) activity (●), specific LDH activity (○) and cell growth (Δ).

and LDH activity was found to be 30°C, and the maximum growth of 1.90 at A_{650} together with a maximum enzyme activity of 1.84 U per mL. Based on these results, it is concluded that optimum environmental conditions in view of growth and total enzyme activity were at pH 6.5 and 30°C.

Time-course batch growth under optimal conditions : A typical batch growth on 2% fructose medium containing 4% yeast extract and 0.05% KH_2PO_4 at 30°C and pH 6.5 is shown in Figure 3. Exponential growth was evident in an early phase of incubation and the growth was complete after 24 h with a maximum cell growth of 2.2 at A_{650} . Total and specific enzyme activities were found to be 2.08 U per mL and 0.60 U per mg protein, respectively. After the completion of growth due to the shortage of carbon source, there was a loss in the total enzyme activity. It appears from Figure 3 that the total enzyme activity of cell-free extract was closely coupled to growth, suggesting that it is necessary to control the feed rate during a fed-batch culture with strain FFy 111-1 for further improvement of growth.

CONCLUSION

In the current study, various bacterial strains were screened from a variety of environments. Among the isolated strains, *Lactobacillus* sp. FFy 111-1 isolated from *Kimchi* showed the highest LDH activity. Initial characterization of strain FFy 111-1 in the present study suggested that it could be a potential candidate for use as a direct-fed microbial to reduce lactate accumulation in the rumen.

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