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OPTIMISATION OF SIGNIFICANT FEATURES ON LACTIC ACID AND LACTASE ENZYME PRODUCTION IN MINERAL SALTS MEDIUM BY *L. BIFERMENTANS*

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ABSTRACT

The aim of this work was to study the fermentation of lactose substrate in mineral salts medium for the production of L(+) lactic acid and lactase enzyme using Lactobacillus bifermentum. The effect of different process parameters such as various lactose concentration, pH of the medium, temperature and inoculum concentration was monitored to enhance the lactose conversion into lactase and lactic acid. Fermentations were performed in batch mode method with suitable environment. The optimization of the fermentation conditions resulted in significant decrease in fermentation time, besides increase in lactose conversion to lactic acid and lactase. The optimized process conditions resulted in high lactic acid production (1740mg/L) and high lactase enzyme production (2.91ALU) at 0.5% lactose, 1% inoculum, pH6 and temperature 30°C.

INTRODUCTION

Lactic acid ($CH_3CHOHCOOH$) is an invaluable chemical, initially discovered by the Swedish chemist Scheele in 1780, who identified it in sour milk (Benninga, 1990; Altaf and Naveena, 2005). In 1789, Lavoisier named this milk component “acide lactique”, which became the possible origin of the current terminology for lactic acid (Benninga, 1990). In the early 1960s, a method to synthesize lactic acid chemically was developed due to the need for heat-stable lactic acid in the baking industry (VickRoy, 1985).Although lactic acid can be manufactured either *via* chemical synthesis or by a biological approach, a great deal of interest has recently become focused on the biological approach, because the chemical synthesis of lactic acid is associated with several serious problems, including environmental issues and the depletion of petrochemical resources (Datta *et al*.,1995; Wee *et al*.,2004). The biological production of lactic acid *via* microbial fermentation has been studied extensively by a lot of research groups (Hofvendahl and Hahn-Hagerdal, 2000).

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Although racemic DL-lactic acid is always produced by chemical synthesis from petrochemical resources, an optically pure L(+)- or D(–)- lactic acid can be obtained by microbial fermentation of renewable resources when the appropriate microorganism that can produce only one of the isomers is selected (Hofvendahl and Hahn-Hagerdal, 2000). The optical purity of lactic acid is crucial to the physical properties of poly (lactic acid) (PLA) and an optically pure L(+)- or D(–)- lactic acid, rather than racemic DL-lactic acid, can be polymerized to a high crystalline PLA that is suitable for commercial uses (Lunt, 1998; Sodergard and Stolt, 2002). Biotechnological processes for the production of lactic acid usually include lactic acid fermentation and product recovery and/or purification. There have been numerous investigations on the development of biotechnological processes for lactic acid production, with the ultimate objectives to enable the process to be more efficient and economical. Therefore, the biotechnological production of lactic acid has received a significant amount of interest recently, since it offers an alternative to environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources. The lactic acid has been used industrially in various aspects. It has been classified by the US FDA as GRAS (Generally Recognized As Safe) for use

as a food additive and it has been utilized in a broad range of applications in the food, cosmetic, medical and pharmaceutical industries (Datta *et al.,* 1995; Litchfield, 1996; Naveena *et al.*, 2004). Lactic acid plays a vital role in the chemical industry, where it is used as a precursor for the syntheses of ethyl lactate, propylene oxide, propylene glycol, acrylic acid, 2,3-pentanedione and di-lactide (Varadarajan and Miller, 1999; Wee *et al.*,2004). Moreover, lactic acid is used commercially in the processed meat and poultry industries, to provide products with an increased shelf life, enhanced flavour and better control of food-borne pathogens. Due to the mild acidic taste of lactic acid, it is also used as an acidulant in salads and dressings, baked goods, pickled vegetables and beverages. Lactic acid is used in confectionery, not only for flavour, but also to bring the pH of the cooked mix to the correct point for setting. The advantages of adding lactic acid in confectionery include its low inversion rate, ease of handling and ability to produce clear candies.

Another potential application of lactic acid in the food industry is the mineral fortification of food products. Lactic acid offers natural ingredients for cosmetic applications. Although primarily used as moisturizers and pH regulators, they possess multiple other properties such as antimicrobial activity, skin lightening and skin hydration. The moisturizing effect is related directly to lactate's water retaining capacity and the skin-lightening action of lactic acid is produced by the suppression of the formation of tyrosinase. Since they are natural ingredients of the human body, lactic acid and its salt fit perfectly into the modern trend towards natural and safer formulations and they produce such effects as skin lightening and rejuvenation, which makes them very useful as active ingredients in cosmetics. Lactic acid is also used in the pharmaceutical industry as an electrolyte in many parenteral/ intravenous solutions that are intended to replenish the bodily fluids or electrolytes. Examples include Lactated Ringer's or Hartmann's solutions, CAPD (continuous ambulatory peritoneal dialysis) solution and dialysis solution for conventional artificial kidney machines. Moreover, lactic acid is used in a wide variety of mineral preparations, which include tablets, prostheses, surgical sutures and controlled drug delivery systems.

Lactic acid and its salt are used increasingly in various types of chemical products and processes. In this category of applications, lactic acid functions as a descaling agent, pH regulator, neutralizer, chiral intermediate, solvent, cleaning agent, slow acid-release agent, metal complexing agent, antimicrobial agent and humectants. Natural lactic acid has an emerging use as an excellent and safe solvent, which is alternative in many fine mechanical cleaning applications. Due to the high solvency power and solubility of lactic acid, it is an excellent remover of polymer and resins. It is available with an isomeric purity greater than 98 % and is suitable as a starting material in the production of herbicides or pharmaceuticals. Since lactic acid offers better descaling properties than conventional organic descalers do, it is often used in many decalcification products, such as bathroom cleaners, coffee machines and toilets. Ethyl lactate is used in many anti-acne preparations, because it combines excellent solvency power against oils and polymeric stains, with no environmental impact and toxicological effects. Currently, lactic acid is considered the most potential feedstock monomer for chemical conversions, because it contains two reactive functional groups, a carboxylic group and a hydroxyl group. Lactic acid can undergo a variety of chemical conversions into potentially useful chemicals, such as propylene oxide (*via* hydrogenation), acetaldehyde (*via* decarboxylation), acrylic acid (*via* dehydration), propanoic acid (*via* reduction), 2,3-pentanedione (*via* condensation) and di-lactide (*via* self-esterification) (Varadarajan and Miller,1999). Lactic acid has recently received a great deal of attention as a feedstock monomer for the production of PLA, which serves as a biodegradable commodity plastic. The optically pure lactic acid can be polymerized into a high molecular mass PLA through the serial reactions of polycondensation, depolymerization and ring-opening polymerization (Sodergard and Stolt, 2002). The resultant polymer, PLA, has numerous uses in a wide range of applications, such as protective clothing, food packaging, mulch film, trash bags, rigid containers, shrink wrap and short shelf-life trays (Drumright *et al.*, 2000; Vink *et al.*, 2003). The recent huge growth of the PLA market will stimulate future demands on lactic acid considerably (Datta *et al.*, 1995; Drumright *et al.*, 2000).

However, the global consumption of lactic acid is expected to increase rapidly in the near future. Nature Works LLC, a major PLA manufacturer established in the US, expects that the global PLA market may increase to 500 000 (metric) tonnes per year by 2010. The worldwide demand for lactic acid is increasing substantially at present, as it can also be employed as a monomer for the synthesis of biodegradable poly- (lactic acid) (PLA), a sustainable bioplastic material (Datta *et al.*, 1995; Litchfield, 1996). Cargill Dow LLC, the primary US manufacturer of PLA, has reported that the global PLA market might expand to 500 000 tonnes per year by 2010. Therefore, considerable increases in the worldwide demand for lactic acid are definitely expected in the coming years. Milk, a vital nutrient for all living beings, contains lactose, proteins, fat, vitamins and minerals such as calcium and phosphorus. Among these, lactose, as the main carbohydrate in milk, is a type of sugar that is the major carbon source during growth (Mahoney, 1985). Lactase (EC 3.2.1.23) hydrolyses lactose into glucose and galactose (Szczodrak and Wiater, 1998; Nagy *et al.*, 2001). In case of shortage of lactase in small intestine, lactose cannot be hydrolysed, which is called lactose intolerance (Paige and Davis,1985; Daniel *et al.*,2002). For this reason, majority of the world population encounters problems due to their consumption of foods containing high amount of lactose. Therefore, pre-processing of milk and dairy products with lactase to hydrolyse lactose is necessary in order to eliminate this disadvantage (Pivarnik *et al.*, 1995).

Lactase (β-Galactosidase: EC3.2.1.2.3) enzyme hydrolyzes lactose, the main carbohydrate in milk, into glucose and galactose, which can be absorbed across the intestinal epithelium (Kosaric and Asher, 1995; Vasiljevic and Jelen, 2001; Troelsen, 2005). This enzyme is mainly produced by fungi, bacteria and mammals. Consequently, there are many different types of lactase, which vary slightly in terms of their ideal environment, including the pH they need to live. This is one of the reasons why lactase supplements come in different forms, like pills and liquids. The type used in making pill supplements can generally live in a higher pH environment than the one used in making drops, which makes it more

suitable for going directly into the acidic environment of the stomach. Some of the most common types of this enzyme are *Kluyveromyces lactis*, *Kluyveromyces fragilis* and *Aspergillis oryzae.* Almost everyone is born producing lactase, since it is needed for digesting breast milk. About two-thirds of people gradually make less of it as they age and may stop making it entirely as adults. This leads to adult-type hypolactasia, or lactose intolerance (Heyman, 2006). Whether or not someone will develop lactose intolerance is usually genetically determined and can be influenced by ethnicity. For example, about 90% of Chinese, 70% of African-Americans and 50% of Spaniards are lactose intolerant, but only about 10% of Caucasian Americans, 10% of Tutsis and less than 5% of Swedes are. It's thought that this reflects evolutionary changes in people who lived in societies with cows or dairy industries.

Rarely, a person may be born with lactase deficiency, which is also known as a congenital absence, because of a mutation of the specific gene that is responsible for its production. This is usually identified shortly after birth and can be treated or mitigated. Diseases which destroy the lining of the small intestine may also cause a deficiency by destroying the cells' ability to produce the enzyme. There is no treatment that can enhance the body's ability to produce this enzyme. The symptoms of lactose intolerance can be controlled easily through diet and lifestyle changes though. Avoiding milk, drinking lactose-reduced milk or using supplements can help prevent discomfort and gastrointestinal symptoms. Though the standard dosage of tablets is about 6000 to 9000 IU, people may need to take more or less depending on the severity of the lactose intolerance, the amount and type of food eaten and their personal gastrointestinal health, among other things. There are no known side effects or drug interactions with this substance, but people may have individual sensitivities (Prenosil *et al.*, 1983).

Besides being used for supplements, lactase is used commercially to reduce the amount of lactose in dairy products before they're consumed, as in the case of low-lactose milk. The way it modifies lactose changes the way that dairy products taste and act, so it's added to some dairy-based syrups and flavoured milks to make them sweeter, longer lasting, and less susceptible to bacterial damage. It's also used to make the texture of some ice creams smoother. In laboratories, lactase is commonly used in screening for several types of bacteria.
The bacterial culture Lactobacillus bifermentum MTCC3818 was used for this study. Consortium proved better efficiency lactic acid and lactase production. Hence this paper mainly focused on optimisation for production of lactic acid and lactase enzyme. In this study the medium used was Mineral Salts Medium. The aim of the present paper was to investigate the applicability of the aerobic bacterial culture for high production of lactic acid and lactase enzyme. Attempts were made to optimize the lactose source, temperature, pH values and inoculum concentration to achieve high production of lactic acid and lactase.

MATERIALS AND METHODS

Microorganism and storage

In this study, *Lactobacillus bifermentum* was selected because of its commercial availability, lactic acid producing abilities and physical versatility. *Lactobacillus bifermentum* (MTCC3818) was obtained from Microbial Type Culture Collection Centre, Chandigarh. Cells were grown in 500 ml of MRS (de Mann Rogosa Sharpe) broth. The inoculated flask was incubated on a rotary shaker at 30^{o}C and 120rpm for 15 hrs. Cells were centrifuged at 10,000 rpm for 20 min and washed three times with sterile distilled water, then streaked on nutrient agar or MRS slant and stored at 4^{o}C for further studies.

Preparation of starter culture

A loopful of culture (*Lactobacillus bifermentum*) was inoculated in presterilized 100ml nutrient broth. The flask was kept in a shaker at 120 rpm for 16 to 18 hrs at 30^{o}C. The culture broth was centrifuged at 10000 rpm for 20 min. Cell suspension was prepared using sterile distilled water and adjusted to 0.5 OD using UV Visible spectrophotometer (Model : Cyberlab UV100, USA). One percent (10^4 CFU/ml) of the above suspension was used as inoculum for the production of lactic acid and lactase.

Effect of various lactose concentration in Mineral Salts Medium for the production of Lactic acid and lactase

The mineral salt medium containing (g/l) 1g of potassium dihydrogen phosphate, 1g of dipotassium hydrogen phosphate, 2g of potassium nitrate, 0.5g of ammonium chloride, 0.005g of calcium chloride, 0.1g of magnesium sulphate and 0.05g of sodium silicate was prepared. It was additionally supplemented with various lactose concentrations (0.5, 1, 1.5 and 2%). The media was sterilized at 121^{o}C for 15 min. To it 1ml of *Lactobacillus bifermentum* inoculum containing 10^4 CFU/ml of cells were inoculated and kept in a shaker (120 rpm) at 30^{o}C for three days. The samples were drawn aseptically at every 24 hrs and lactic acid production (acid base titration) and lactase enzyme production (Fcc Alu Analytical Method) were estimated.

Effect of various inoculum concentration in Mineral Salts Medium for the production of Lactic acid and lactase

About 0.5% of lactose was found to be ideal concentration of lactose source for production of lactic acid and lactase. Different inoculum dosage of 0.5, 1, 1.5 and 2% of bacterial cells were inoculated in sterilized mineral salt medium (MSM) supplemented with 0.5% lactose. The flakes were and kept in a shaker (120 rpm) at 30^{o}C. The samples were drawn aseptically every 24 hrs and lactic acid and lactase enzyme production were estimated.

Effect of various pH concentration in Mineral Salts Medium for the production of Lactic acid and lactase

Inoculum of 1% was found to be effective concentration in production of lactic acid and lactase compared to the other concentrations. Hence, 0.5% lactose and 1% inoculum was used for further study. Mineral salt medium (MSM) with various pH (5, 6, 7, 8 and 9) and supplemented with 0.5% lactose was prepared and sterilised. The medium was inoculated with 1% of bacterial culture and kept in a shaker (120 rpm) at 30^{o}C. The samples were drawn aseptically every 24 hrs and lactic acid and lactase enzyme production were estimated.

Effect of various temperature for the production of Lactic acid and lactase using Mineral salt medium (MSM)

The pH 6 was found to effective for production of lactic acid and lactase compared to the other concentrations. Hence, 0.5% lactose, 1% inoculum and pH 6 were used for further study. Mineral salt medium (MSM) supplemented with 0.5% lactose with pH 6 was prepared and sterilised. The medium was inoculated with 1% of bacterial culture and kept in a shaker (120 rpm) at various temperature (30, 35 and 40^{o}C), to find out the optimum temperature required for the production of lactic acid and lactase. Every 24hrs the samples were drawn and lactic acid and lactase enzyme production were estimated.

RESULTS AND DISCUSSION

Production of lactic acid and lactase in mineral salts medium amended with lactose

Production of lactic acid and lactase enzyme was found to be maximum ie., 1200 mg/L and 1.57ALU respectively with lactose concentration of 0.5% compared to the other concentration (Fig. 1 and 2). About 180mg/L and 0.398 ALU of lactic acid and lactase was found to be the least production with lactose concentration of 2%.

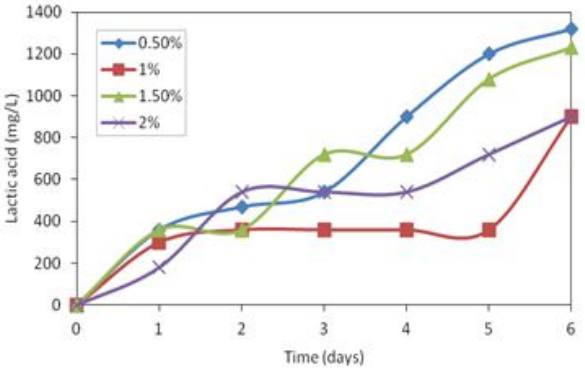

Fig. 1. Production of lactic acid in MSM containing various lactose concentrations

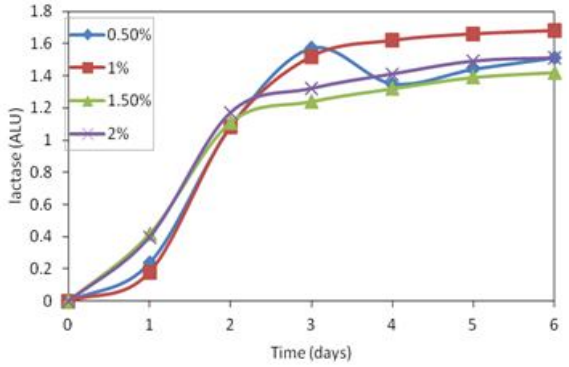

Fig. 2. Production of lactase in MSM containing various lactose concentrations

Production of lactic acid and lactase in MSM with various inoculums concentrations

In the case of various inoculum concentration production of lactic acid and lactase was found to be maximum 1540mg/L and 2.91ALU respectively with inoculum concentration of 1% (Fig. 3 and 4). In the lower inoculum concentration 0.5% and higher inoculum concentration 2% the lactic acid and lactase production was very low ie., 360mg/L and 0.1ALU respectively.

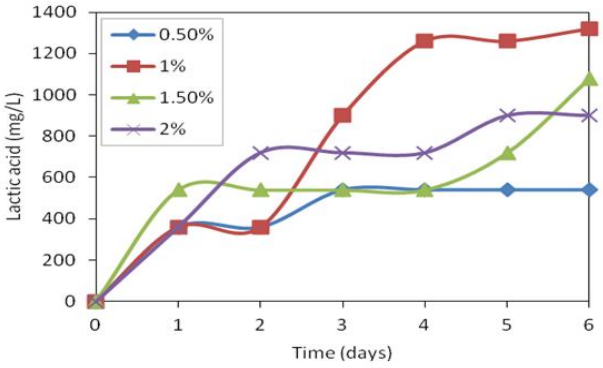

Fig. 3. Production of lactic acid in MSM containing various inoculum concentrations

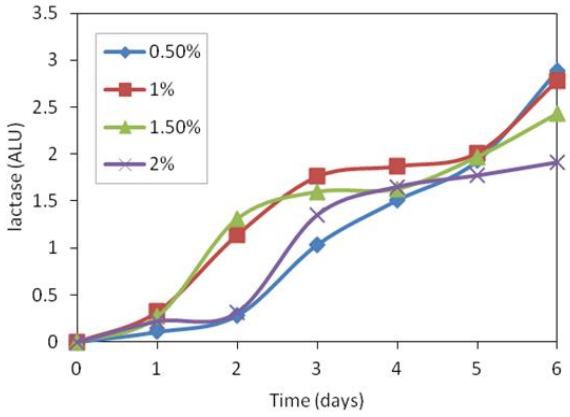

Fig. 4. Production of lactase in MSM containing various inoculum concentrations

Production of lactic acid and lactase in MSM at various pH

In the study of optimisation with various pH concentration the result was very well apparent that production of lactic acid and lactase was maximum ie., 1740mg/L and 2.01ALU respectively at pH 6 (Fig. 5 and 6).

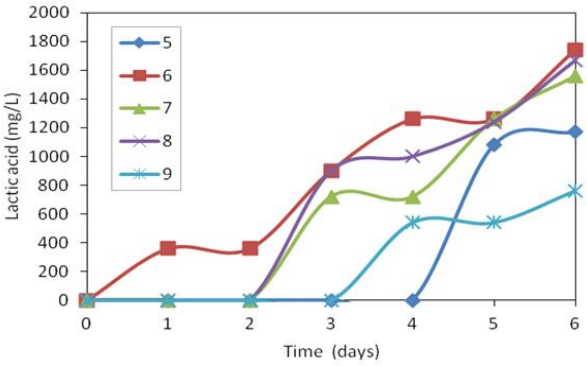

Fig. 5. Lactic acid production in MSM at various Ph

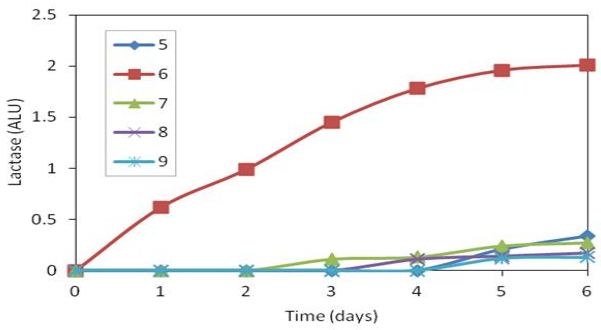

Fig. 6. Lactase enzyme production in MSM at various pH

Whereas in the range closer to this optimum range ie., at pH 5 and 7 the lactic acid production was 1170mg/L and 1260mg/L. At pH 9 the production was 540mg/L which is very low.

Production of lactic acid and lactase in MSM at various temperatures

In the final optimisation with various temperatures it was very clear that lactic acid and lactase was produced only at 30°C. Lactic acid production was 1342mg/L and lactase 2.01 ALU. At 35°C and 40°C no production was observed (Fig. 7,8).

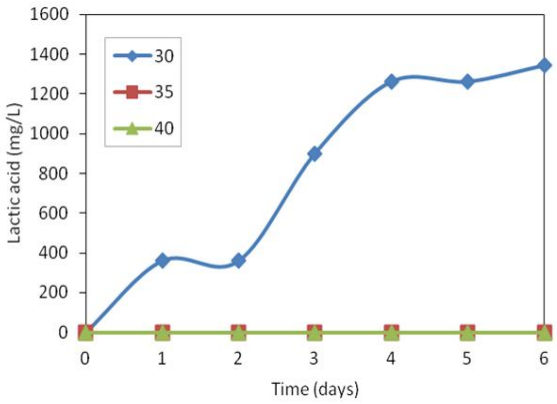

Fig. 7. Lactic acid production in MSM at various temperatures

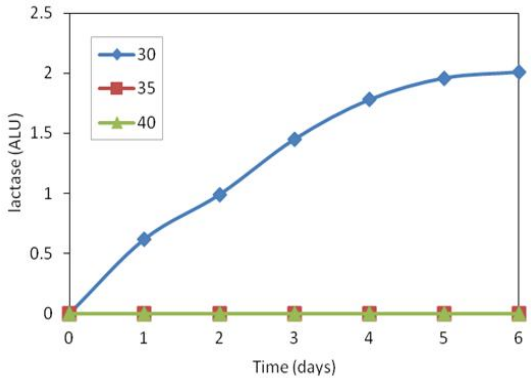

Fig. 8. Lactase production in MSM at various temperatures

Conclusion

In this study, the bacterial consortium *Lactobacillus bifermentum* (MTCC3818) was obtained from Microbial Type Cell Culture Chandigarh. Among the various lactose concentrations, 0.5% was observed to be efficient in producing lactic acid (1740mg/L) and lactase enzyme (2.91ALU) compared to the other. Hence, 0.5% of lactose was used as standard concentration and various inoculum concentrations were experimented. In this study 1% of inoculum was efficient in producing maximum level of lactic acid (1740mg/L) and lactase enzyme (2.91ALU) compared to the other concentration. Further, media standardised with 0.5 % lactose and 1% inoculum was experimented with various pH. The production of lactic acid and lactase enzyme was high in pH6 than the other range. Finally, media prepared with 0.5% lactose, pH 6 and inoculum concentration of 1% was incubated in various temperatures for the study. Efficient lactic acid and lactase enzyme was produced at the temperature 30°C when compared to the other temperatures. Hence, 0.5 % lactose, 1% inoculum, pH 6 and temperature 30°C are found to be the optimised parameter required for the production of lactic acid and lactase enzyme.

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