EFFECTS OF INOCULUM SIZE AND TIME OF INCUBATION FOR Enterococcus faecium NO. 78 ON LACTIC ACID PRODUCTION

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EFFECTS OF INOCULUM SIZE AND TIME OF INCUBATION FOR
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Effects of Inoculum Size and Time of Incubation for Enterococcus faecium No. 78 on Lactic Acid Production

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ABSTRACT

The effects of different inoculum size and time of incubation for Enterococcus faecium No. 78 on lactic acid production were studied. The experiment was carried out utilizing three different inoculum volumes which were 5% (v/v), 10% (v/v) and 15% (v/v). The effect of incubation time was examined based on cell growth, starch concentration which is not hydrolyzed and glucose consumption within 30 hours. The batch fermentation processes were conducted utilizing gelatinized sago starch at initial pH 6.5, temperature of 30°C and agitation of 200 rpm. The experiment was carried out in shake flask and the pH was uncontrolled throughout the study. Maximum cell growth was obtained in 9 hours incubation time with 95 x 10^8 CFU/ml. Higher lactic acid production was obtained at 2.94 g/L with 10% inoculum volume. Lower lactate concentration was observed at 5% (v/v) and 15% (v/v) inoculum volumes at 2.17 g/L and 1.48 g/L, respectively. Overall, 10% (v/v) inoculum volume was observed as optimum inoculum size with higher lactic acid production.

Keywords: Enterococcus faecium No. 78; batch fermentation; sago starch; inoculum volume; lactate concentration.

ABSTRAK

Kesan perbezaan saiz nekulum dan masa penggeraman Enterococcus faecium No. 78 terhadap penghasilan laktat telah dilakukan. Uji ini telah dilaksanakan pada tiga isipada nekulum yang berbeza iaitu 5% (v/v), 10% (v/v) dan 15% (v/v). Kesan terhadap masa penggeraman telah diuji berhubungkan pertumbuhan sel kepekatan kanji yang tidak dihidrolisiskan dan penggunaan glukosa sehingga 30 jam. Proses fermentasi berkelompok dilakukan menggunakan nekulum yang telah digelatiniser pada formula pH 6.5, suhu 30°C dan kocakan pada 200 rpm. Uji ini dilakukan menggunakan kelangsungan dan pH tidak dikawal. Pertumbuhan sel yang maksimum diperoleh semasa jam penggeraman ke-9 dengan jumlah sel 95 x 10^8 CFU/ml. Penghasilan laktat yang paling tinggi diperoleh dengan menggunakan isipada 10% (v/v) dengan laktat sebanyak 2.94 g/L. Penghasilan laktat lebih rendah pada isipada nekulum 5% (v/v) dan 15% (v/v) dengan jumlah masing-masing 2.17 g/L dan 1.48 g/L. Secara keseluruhannya, isipada nekulum yang optimum adalah 10% (v/v) dengan penghasilan laktat paling tinggi.

Kata Kunci: Enterococcus faecium No. 78; fermentasi berkelompok; kanji sago; isipada nekulum; kepekatan laktat.
CHAPTER 1
INTRODUCTION

Lactate fermentation, which is an anaerobic process, is characterized by product inhibition, which affects cell growth and metabolism hence limiting the amount of product formed and increasing the downstream processing cost (Senthuran et al., 1997). Lactate is converted back to pyruvate when conditions are aerobic and there is no net carbon loss.

Much attention has been paid recently to the production of L (+)-Lactic acid from biomass as a monomer of polylactic acid (PLA), a novel biodegradable polymer, which is also used as a carrier system in drug formulations among others. L (+)-lactic acid is widely used in the food industry as preservative or taste-enhancing additive and also in the pharmaceutical and chemical industries (Datta, 1995). Lactic acid fermentation also has some other distinct advantages, for example the food becomes resistant to microbial spoilage and to development of toxins. Acid fermentation also modifies the flavour of original ingredients and often improves the nutritive value. However, direct fermentation of sago starch to valuable products has not fully been explored by the industry (Ariff et al., 2002).

Pearman (2002) reported in a fermentation process, the inoculum preparation step is very important and will affect the production of lactic acid. Inoculum is a medium containing organisms, usually bacteria or a virus, which is introduce into cultures or living organisms. The effects can be identified and studied based on production of lactate, biomass formation and glucose utilization.
An alternative strategy that involves direct conversion of starch to lactic acid using both amylolytic and acid producing organisms would considerably reduce the cost of production, as liquefaction and saccharification processes will be eliminated (Vishnu et al., 2002). Dulce et al. (2001) reported that fermentation of lactic acid utilizing sago starch can be produced directly using Lactococcus strains. As explained by Manero and co-workers (1999), optimum pH and temperature on direct L-lactic acid production from sago starch was carried out with Enterococcus faecium No. 78.

In this study Enterococcus faecium No. 78, an amylolytic lactic acid bacterium will be used as a microorganism for producing lactate directly utilizing sago starch as a carbon source. The effects of Enterococcus faecium No. 78 on inoculum size and time of incubation will be studied on the production of lactic acid.

OBJECTIVES

The objectives of this study are to determine the:

- optimum inoculum sizes of Enterococcus faecium No. 78.
- time of incubation for Enterococcus faecium No. 78 utilizing sago starch as the sole carbon source for lactic acid production.
CHAPTER 2
LITERATURE REVIEW

2.1 Lactic acid

Lactic acid has been first introduced to us as early as 1780 by Scheele as a sour component of milk. Lactic acid (2-hydroxypropanoic acid) is the most widely used occurring natural organic acid. It was first produced commercially by Charles E. Avery at Littleton, Massachusetts, USA in 1881 (Vickroy, 1985). The lactic acid production from various crude starchy substrates further reduces the production cost (Vishnu et al., 2002).

It has numerous applications in the food, pharmaceutical, leather and textile industries and also as a chemical feedstock. The increasing applications of lactic acid in newer avenues like biodegradable plastics, medical care, agriculture and also as green solvents, had led to constant increase in its demand (Datta et al., 1993; Litchfield, 1996). Lactic acid has the potential of becoming a very large volume, commodity-chemical intermediate produced from renewable carbohydrates for use as feedstock for biodegradable polymers, oxygenated chemicals, and plant growth regulators (Datta et al., 1995).

\[ \text{CH}_3\text{CHOHCO}_2\text{H} \] is a colorless liquid organic acid. Lactic acid among acidulants has proved to be a most effective, tasteful and strong preservative of food products. It’s consumption covers a vast range of medicines, drinks, food products of different types, plastics and leather tanning. There are several merits of lactic acid from a health point of view, it does not create any foreign element in food, it can be used in calcium purpose products, also being easily
soluble and it is used in sports drinks. It can also be used for pH regulation and with its distinct preservative action, it also regulates microflora.

Although it can be prepared by chemical synthesis, production of lactic acid by fermentation of glucose and other substances is a less expensive method. Chemical synthesis gives racemic lactic acid, whereas fermentation technology can produce desired stereoisomers such as L (+)-lactic acid or D (-)-lactic acid or a racemic mixture of lactic acid using hemolytic organisms (Datta et al., 1993; Litchfield, 1996). Lactic acid is miscible with water or ethanol. Specifically, lactic acid is a carboxylic acid and an alcohol. The protein in milk is coagulated (curdled) by lactic acid.

Calcium lactate, a soluble lactic acid salt, is used as a source of calcium in the diet. Lactic acid is also produced in the muscles during intense activity. During anaerobic condition, the six-carbon glucose molecule is only partly broken down by certain bacteria to two molecules of three-carbon sugar called lactic acid. Lactic acid has also shown promise in inhibiting mycotoxigenic fungi, and foot-and-mouth virus in sheep’s dung.
2.2 *Enterococcus faecium* No.78

Lactic acid bacteria perform an essential role in the preservation and production of wholesome foods ranging from fermented fresh vegetables such as cabbage (sauerkraut) and cucumbers to fermented cereals yogurt, sour dough bread, and fermented milk. Lactic acid bacteria are gram positive, nonsporulating, rods and cocci that produce lactic acid as a major fermentation product. Members of these group lack porphyrins and cytochromes, do not carry out electron transport phosphorylation, and hence obtain energy only by substrate level phosphorylation.

All lactic acid bacteria grow anaerobically. Unlike many anaerobes, however, most lactic acid bacteria are not sensitive to oxygen and can grow in it's presence as well as in its absence. High acid tolerance is a distinctive feature of LAB, and this allows the fermentation process to proceed almost free of contamination (Hipolito, 2001). Thus they are aerotolerant anaerobes. They usually have only limited biosynthetic ability, and their complex nutritional requirements include needs for amino acids vitamins, purines and pyrimidines. Fermentation using lactic acid bacteria is consider the most suitable method to produce highly pure L(+) or D(-) lactic acid to meet the critical requirements of the PLA polymerization process (Yin et al., 1998).

They are of two types: 1) homolactic, which includes all members of genera *Peidococcus*, *Streptococcus* and *Lactococcus* and 2) heterolactic including all members of genera *Leuconostoc* and *Lactobacillus* (Madigan et al. 2000). One important difference between subgroups of lactic acid bacteria lies in the nature of products formed from the fermentation of sugars. One group, called homofermentative, produces a single fermentation product, lactic
acid, whereas the other group, called heterofermentative, produces other products mainly ethanol and carbon dioxide as well as lactate. The differences observed in the fermentation patterns are determined by the presence or absence of the enzyme aldolase (Madigan et al., 2000).

Homofermenters lacking aldolase cannot breakdown fructose bisphosphate to triose phosphate. Instead they oxidise glucose 6-phosphate which is broken down to triose phosphate and acetylphosphate by means of enzyme phosphorylase. In heterofermenters triose phosphate is converted ultimately to lactic acid with the production of 1 mol of ATP, while the acetyl phosphate accepts electrons from NADH generated during the production of pentose phosphate and is thereby converted to ethanol without yielding ATP. Because of this, heterofermenters produce only 1 mol of ATP from glucose instead of 2 mol produced by homofermenters. Also heteroferments decarboxylate 6-phosphogluconate, they produce carbon dioxide as a fermentation product, whereas homofermenters produce little or no carbon dioxide. Therefore one simple way of detecting a heterofermenter is to observe the production of carbon dioxide (Madigan et al., 2000).
2.3 Sago Starch

Starch is considered to be one of the most abundant plant products and a major source of energy in the human diet. The sago palm belongs to the Lepidocaryoid subfamily of the Arecaceae (Palmae). Sago palm or Metroxylon spp., better known as 'rambutia' is commonly distributed in Southeast Asia Region. It is planted mainly along coastal area of Mukah, Oyah and Dalat in the district of Sibu. The true sago is a hapaxantie (monocarpic or once-flowering) and soboliferus (tillering or suckering) feather-leaf palm of the Lepidocaryoid subfamily (Kiew, 1977).

Sago is potentially a very important starch producing tree which grows well in the swampy lowlands with minimum care (Othman, 1991). Sago (Metroxylon sago) is a giant semi-cultivated palm of swampy areas with flowering stems up to 26 metres tall. In drier locations occasionally the palm is harvested and sometimes also cultivated for the starch it accumulates in its trunk. If not harvested, most starch is ultimately used to form the majestic inflorescence and the seeds after which the trunk dies (Flach, 1983). Since sago starch is of large in granules size, it can possess high swelling power and provide high viscosity, the properties which are exploited when utilizing the starch as a thickening agent (Sriroth, 1999). It has been estimated that Sarawak has been producing about 55,000 tones starch each year (Tie et al., 1991). The biggest sago plan in Sarawak is located in Mukah that is about 75% (Bujang and Ahmad, 2000). Sarawak has been the principal exporter of sago starch to the world market.
Some of the important species widely used in sago starch production includes *M. longispinum*, *M. sylvestre, M. microcanthum, M. sago* and *M. rumphii*. Sago palm is an important resource especially to the people in rural areas due to it having various uses especially in the production of starch either as sago flour or sago pearl (Ahmad *et al.*, 1999). Sago will remain an important source of starch for local food industries especially for manufacturing monosodium glutamate and glucose. Less energy is required for the extraction of starch from sago palm than other crops such as root crops and cereals. Further, the sago starch contains less contaminant such as sugar and protein than other crops. Sim (1977) stated sago starch has several advantages over other starches because it produces pastes of lower viscosity at a given concentration than such pastes from maize and potato. This starch, when extracted from the felled palm before it flowers, is the commercial source of sago, and also a valuable food supply in several parts of the Malay Archipelago and Polynesia (Barrau, 1959).
2.4 Inoculum- Seed culture

Minimum inoculum size is the critical volume of inoculum required to initiate culture growth, due to the diffusive loss of cell materials into the medium. The subsequent culture growth cycle is dependent on the inoculum size, which is determined by the volume of medium and size of the culture vessel. Inoculum is a medium containing organisms, usually bacteria or a virus, which is introduce into cultures or living organisms.

Inoculum is defined as a culture medium to initiate growth when microbes are introduced to it (Tortora et al., 2004). According to Martin and Hine (2000), inoculum means as a small amount of material containing bacteria, viruses, or other microorganisms or any other type of cell into a culture medium so that the cells can grow and proliferate. When a liquid nutrient medium is inoculated with a seed, the organisms selectively take up dissolved nutrients from the medium and convert them into biomass. The lag phase occurs immediately after inoculation and is a period of adaptation of cells to a new environment.

Microorganisms are reorganizing their molecular constituents when they are transferred to a new medium. When the inoculum is small and has a slow fraction of cells that are viable, there may be a pseudo-lag phase, which is not because of adaptation, but as a result of small inoculum size. The age of the inoculum culture has a strong effect on the length of lag phase (Shuler, 1992).
The common inoculum size that usually used in lactate fermentation is 10% (volume/volume) and 5% (volume/volume) of the working volume. The 5% (volume/volume) inoculum will be in a fermentation process (Ishizaki and Ohta, 1989).

2.5 Batch Fermentation

Batch fermentations are carried out in fermentors, where the feed is introduced only once before the fermentation is initiated. Batch system is ideal, in terms of its low maintenance and very low risk of contaminations. Its operation however requires high degree of labour to start, end and restart the new fermentation. In the fermentation industries batch fermentation is used for the production of biomass, primary metabolites and secondary metabolites. Most fermentation operations are conducted batchwise even when the scale of production is quite large.

Batch processing is flexible and readily adaptable to different needs; however, because of ever changing conditions, engineering analysis of batch operations is more difficult than analysis of continuous one (Sharatt, 1999). In batch processes, cell nutrients required during one minute of cultivation, except for molecular oxygen in an aerobic process and ammonia or other chemicals for pH adjustment, are added to the medium before cultivation started, and the broth containing the product is withdrawn only at the end of each batch run (Yamane, 1995).
CHAPTER 3
MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Sago Starch
Commercial food grade starch powder was used in this experiment. The starch powder must be free of moisture in an airtight container. This sago starch powder was obtained from local supermarket.

3.1.2 Enterococcus faecium No. 78
The microorganisms Enterococcus faecium No. 78 was used in this experiment. The prepared stock culture was kept in glycerol stock at temperature of -84°C. The stock culture was revived in MRS (DeMan Rogosa Sharpe)-sago starch and was incubated at 30°C for between 18 and 24 hours.

3.1.3 Enzymes for Hydrolysis
The enzymes used for sago starch hydrolysis were Thermamyl-120L (thermostable amylase from Bacillus licheniformis, 120 KNU/g) and Dextrozyme (a mixture of glucoamylase from Aspergillus niger and pullulanase from Bacillus acidopullulyticus, 225 AGU/mL) supplied by Novo Nordisk.
3.1.4 Culture Media

MRS-sago starch agar was used to culture *Enterococcus faecium* No. 78. The MRS-sago starch media with 10g/L starch concentration was used, for inoculum preparation. A 10% (volume/volume) of inoculum was utilized for all batch fermentation trials.

3.1.5 Fermentation System

The fermentation was carried out in 500 ml shake flask with working volume of 300 ml for 30 hours with mild agitation (200 rpm). In order to maintain a homogenous culture, the initial pH was controlled at 6.5.
3.2 METHODS

3.2.1 Activation of *Enterococcus faecium* No.78

The strain of *Enterococcus faecium* No.78 was revived in MRS-sago starch media at the temperature of 30°C for 18 to 24 hours without agitation. The samples were taken every six hours to detect the growth phase of the microorganisms.

3.2.2 Enzymatic Hydrolysis of Sago Starch

The pH level of 9mL sample broth was adjusted to 6.5 using 1M NaOH and 1M H₂SO₄. Liquefaction process was carried out by adding 0.2 μL of Termamyl-120L and 2mg ion Ca⁺⁺ to the sample for enzymatic reaction at 80°C-90°C for 2 hours. After that, saccharification step was carried out by adding 0.2 μL Dextrinase at pH 4.5 and was incubated at 60°C-65°C for further 2-4 hours. After enzymatic reaction, centrifugation was done for removal of insoluble materials at 13,000 rpm for 15 minutes at 4°C. Determination of glucose in sample was carried out by DNS method (Miller, 1959). Details of this process have been reported earlier (Bujang et al., 1999).

3.2.3 Inoculum Preparation (Incubation Time)

For batch fermentation trial utilizing shake flask, 10% (volume/volume) inoculum was used for the first trial. The inoculum was incubated at 30°C for 24 hours. An aliquot of 10 ml of sample was taken out from shake flask every 3 hours manually and stored at 4°C before further analysis. From this result, the incubation time with the highest cell growth was used to study the different inoculum sizes utilizing 5% (volume/volume) inoculum and 15%
(volume/volume) inoculum. The study on incubation time of inoculum was carried out using a 1000 ml shake flask with 450 ml working volume.

3.2.4 Inoculum Preparation (Size)

The experiment of the first trial was repeated with 5% (v/v) inoculum volume. In the next experiment, the 15% (v/v) inoculum volume was carried out with the same incubation time. From this result, the incubation time with the highest lactate production was determined. The study on different inoculum sizes is carried out using a 500 ml shake flask.

3.2.5 Batch Fermentation System

Shake flasks were used for batch fermentation trials and the fermentation process was conducted in 500 ml shake flasks. The fermentation was conducted with initial pH 6.5, temperature of 30°C.

3.2.6 Sampling

Every 6 hours started from 0 hour up to 30 hours, 5 ml of the sample was taken out from the shake flask under sterile condition in order to determine the glucose consumption, cell growth, lactic acid production and pH reading. Samples were stored at 4°C before the analysis process.
3.2.7 Analytical Techniques

3.2.7.1 Determination of Bacterial Growth by Colony Forming Units

Colony forming units was performed to investigate the increase of colony forming unit in the batch fermentation. On 1 ml of the sample was added to 9 ml of 0.1% peptone water. Serial dilution will be done until appropriate dilution. 0.1 ml of the $10^{-5}$ until $10^{-10}$ dilution was pipetted on the MRS surface and spread uniformly on the surface by spreader. Then, the agar plates were incubated for 24 to 48 hours. The colonies formed on the surface were counted. The bacterial count was converted to log10.

3.2.7.2 Reducing Sugar Analysis

DNS method for reducing sugar was discovered by Miller in 1959 to investigate the utilizing of glucose by the microorganisms. Reducing sugar was analyzed using the dinitrosalicylic acid (DNS) method (Miller, 1959). DNS reagent was prepared according to the percentage of each chemical: 1% DNS powder, 0.2% phenol, 0.05% Sodium sulphite and 1% Sodium hydroxide were dissolved in distilled water and was stirred with a strong magnetic stirrer. After that, 1% DNS powder and 0.2% phenol were dissolved into NaOH solution. Prepared DNS reagent was kept in reagent bottle to avoid crystallization or any chemical reactions. Meanwhile, 0.05% Sodium sulphite was added to this just prior to use.