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Simultaneous Saccharification and Fermentation of Cassava Bagasse for L-(+)-Lactic Acid Production Using *Lactobacilli*

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Abstract

Saccharification and fermentation of cassava (*Manihot esculenta*) bagasse was carried out in a single step for the production of L-(+)-lactic acid by *Lactobacillus casei* and *Lactobacillus delbrueckii*. Using 15.5% w/v of cassava bagasse as the raw material, a maximum starch to lactic acid conversion of 96% was obtained with *L. casei* with a productivity rate of 1.40 mg/mL·h and maximum yield of 83.8 mg/mL. It was 94% with *L. delbrueckii* with a productivity rate of 1.36 mg/mL·h and maximum yield of 81.9 mg/mL. Supplementation of bagasse with 0.01% w/v $MnCl_2$ showed positive influence on the lactic acid production by *L. casei*.

Index Entries: Cassava bagasse; L-(+)-lactic acid; *lactobacilli*; simultaneous saccharification; fermentation.

Introduction

Lactic acid is widely used in food and nonfood industries, such as cosmetics, pharmaceuticals, textile, leather, and other chemical industries, because of the versatile applications of it and its salts. Currently the demand of lactic acid is increased as a result of the environmentally friendly nature of its polymer, polylactic acid *(1–5)*. Lactic acid is commercially produced by chemical synthesis and fermentation. By microbial fermentation, we can

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Job: ABAB 134
Chapter: Nampoothiri (05-0090)
Pub Date: 2006
Operator: KB
Date: 5/06
Revision: 1st galleys

produce the desired isomer, whereas chemical synthesis yields only the racemic mixture *(3,5)*. Lactic acid produced from cheaper starchy substrate can replace the refined sugars *(6)*. Conventional lactic acid production from starchy materials, such as corn, barley, or rice, requires gelatinization, liquefaction, and saccharification before fermentation by lactic acid bacteria. But these processes are not economical because of the high-energy needs for the liquefaction *(7)* and because the production of lactic acid will be inhibited when the sugar concentration is high in the medium. The microbial fermentation coupled with enzymatic hydrolysis of starch has many benefits. This process can decrease the inhibition as a result of the high glucose accumulation and also reduces the reactor volume and the cost of the process *(2)*. Some of the researchers tested simultaneous saccharification and lactic acid production from cellulose *(8,9)*. This can be done using lactic acid producing fungi like *Rhizopus (10)*. But the conversion efficiency of the fungus is less than that of bacteria *(11)*. Linko and Javanainen *(7)* had reported the simultaneous saccharification and fermentation of barley starch for lactic acid production using *Lactobacillus. casei* with the help of starch hydrolyzing enzymes.

Cassava (*Manihot esculenta*) ranks the world's sixth most important food crop and is the basic food for more than seven hundred million people in several countries *(12)*. The cassava, or manioc, is a woody perennial shrub of the spurge family that is extensively cultivated as an annual crop for its edible starchy tuberous root. Industrial application of cassava generates a large quantity of solid bagasse and it is generally discarded in the environment as a landfill without any treatment. Its disposal is a serious concern to the environment *(12,13)*. Cassava bagasse is a fibrous residue, which contains about 52% starch on a dry weight basis *(14)*. In this work, we tried a single-step conversion of the cassava bagasse for the production of L-(+)-lactic acid using two *lactobacilli* namely *Lactobacillus delbrueckii* and *L. casei* and compared their nutritional and other physico-chemical requirements in the production.

Materials and Methods

Microorganism

Homofermentative *L. delbrueckii* NCIM 2025 producing L-lactic acid was procured from National Collection of Industrial Microorganism, National Chemical Laboratory, Pune, India and *L. casei* NCIMB 3254 was obtained from National Collection of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland. Strains were maintained as stab culture at 4°C on MRS agar and when required the strains were revived by propagation at 37°C for 18 h in MRS broth.

Enzymes

For the enzymatic hydrolysis of cassava bagasse, commercially available (Rashesh and Co., Mumbai, India) α-amylase (Termamyl, 5000 IU / mL) and glucoamylase (AMG, 2000 IU / mL) procured were used.

Job: ABAB 134
Chapter: Nampoothiri (05-0090)
Pub Date: 2006
Operator: KB
Date: 5/06
Revision: 1st galleys

Saccharification and Fermentation

Unless it is otherwise emphasized, the production medium was made by supplementing the raw cassava bagasse (15.5% w / v) in 100 mL distilled water with yeast extract (0.5% w / v) and ammonium chloride (0.5% w / v). Tween-40 (0.4% v / v) was added as surfactant and $CaCO_3$ (4% w / v) was added for buffering (to keep the medium pH between 5.5–6.5). Fermentation medium was autoclaved at 121°C (15 lb) for 15 min. Subsequently, the sterile medium was inoculated with 2.5 mL preparatory culture (18 h, CFU / mL 10^9) of *L. delbrueckii* and *L. casei*. Sufficient amounts of both starch-hydrolyzing enzymes were also added to the inoculum. After thorough mixing, the flasks were incubated in static condition at 37°C in an environmental chamber (Sanyo, Japan) for 3 d.

Optimization of Parameters for Lactic Acid Production

The amount of reducing sugar available will depend upon the amount of initial cassava bagasse concentration subjected for saccharification and fermentation. In order to optimize it, different amounts of bagasse (2–17%, w / v) were tried for each organism. Similarly effective conversion depends on the sugar consumption rate and it indirectly depends on the biomass. Because the amount of inoculum is critical in deciding the biomass formation different volumes (1, 1.5, 2, 2.5, 3, 3.5, and 4% [v / v]) of 18 h old cultures were tested; each culture was diluted to approx 10^9 cells per 1 mL. To optimize the incubation time for complete utilization of sugar and also for the maximum accumulation of lactic acid, the inoculated flasks were incubated for different time intervals (12, 24, 36, 48, 60, 72, and 84 h). Samples were withdrawn at regular intervals and the level of sugar and lactic acid were determined. Apart from the carbon source, the organism is in need of a readily available nitrogen source. Ammonium chloride at different levels (0.25, 0.5, 1, 1.5, 2, and 2.5% [w / v]) was supplemented to optimize its level. Because lactobacilli are known as fastidious organisms, yeast extract was shown to have good influence on their growth as it contains many vitamins, amino acids, and other nutrients. Thus different concentrations of yeast extract (0.25, 0.5, 1, 1.5, 2, 2.5, and 3% [w / v]) were tested to achieve maximum growth and product yield. To study the influence of temperature on productivity, fermentation experiments were carried out at different temperatures (30, 37, 42, and 51°C). Other parameters studied include the effect of manganese chloride (0, 0.01, 0.025, and 0.05% [w / v]) on lactic acid production and the amount of $CaCO_3$ requirement (1, 2.5, 4, 5.5, and 7% [w / v]) for effective buffering of the production medium. For comparison, a control flask without $CaCO_3$ was included. In order to see the effect of $MnCl_2$, the ammonium chloride concentration in all the cases, including the control, was reduced from 0.5 to 0.4% (w / v) to avoid excess chloride.

Analysis

Samples were withdrawn at desired intervals, treated, and diluted with *1M* H_2SO_4 to release lactic acid from calcium lactate. Total lactic acid

Job: ABAB 134
Chapter: Nampoothiri (05-0090)
Pub Date: 2006
Operator: KB
Date: 5/06
Revision: 1st galleys

(mg / mL fermentation medium) was estimated according to the colorimetric method of Barker and Summerson *(15)*. The amount of reducing sugar was determined by the dinitrosalicylic acid method *(16)*. Starch was estimated photometrically as described by Nampoothiri et al. *(17)* using an aqueous iodine solution as a reagent; coloration of samples was measured on a UV spectrophotometer (Shimadzu, Japan) at 620 nm. All the experiments were performed in triplicate. The estimation of biomass was interfered by cassava bagasse as the bacterial cells are entrapped and mixed with the bagasse in the fermentation medium. Therefore, the biomass estimation was omitted from the current study and generally the conversion of medium components to biomass was negligible in the case of anaerobic lactic acid fermentation by *lactobacilli*.

Results and Discussion

The optimum temperature and pH for liquefaction and saccharification of gelatinized starch is much different from the fermentation conditions and it can affect the initial growth of the bacteria as well as later growth in terms of lactic acid yield. For example, in a separate enzymatic hydrolysis of starch, the optimum pH and temperature for α-amylase reaction was 5 and 90°C and for glucoamylase it was 4 and 60°C respectively. Under these optimum circumstances a ready release of reducing sugar will take place. However, the strategy we adopted in simultaneous saccharification and fermentation experiments was different. The enzymes were added together with the lactobacilli and the medium pH was 6.0–6.5 and temperature 37°C. Under these conditions the enzymes may not show their optimum level of activity, but a partial level activity is expected. This will help the controlled release of sugars and is very crucial for the fermentation in such a way that even if we use a highly starchy material, feedback inhibition resulting from a higher reducing sugar level will not take place. External supplementation of the enzyme was necessary because both of the strains do not possess any amylolytic activity. Also, under these conditions, the bacteria can grow well to consume the available sugars and to make lactic acid. Interestingly we found that in all the substrate concentrations used for the fermentation, simultaneous saccharification and fermentation had much more productivity than the simple saccharification and fermentation even if the temperature and pH were very far from the optimum (data not shown). Linko and Javanainen *(7)* reported 66% of conversion from starch to sugar during the saccharification at 37°C on barley starch after 24 h. When the temperature rose to 60°C there was only a slight increase, after 24 h the yield was only 69%. In our experiments we performed saccharification as well as the fermentation at pH 6.5 and 37°C.

The initial concentration of cassava bagasse was optimized as 15.5% (w / v) (Fig. 1) as it had the high conversion rate and high production. Further increase of the bagasse amount makes the medium a slurry type, which will readily solidify and negatively influence the mixing of enzymes and

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Job: ABAB 134
Chapter: Nampoothiri (05-0090)
Pub Date: 2006
Operator: KB
Date: 5/06
Revision: 1st galleys

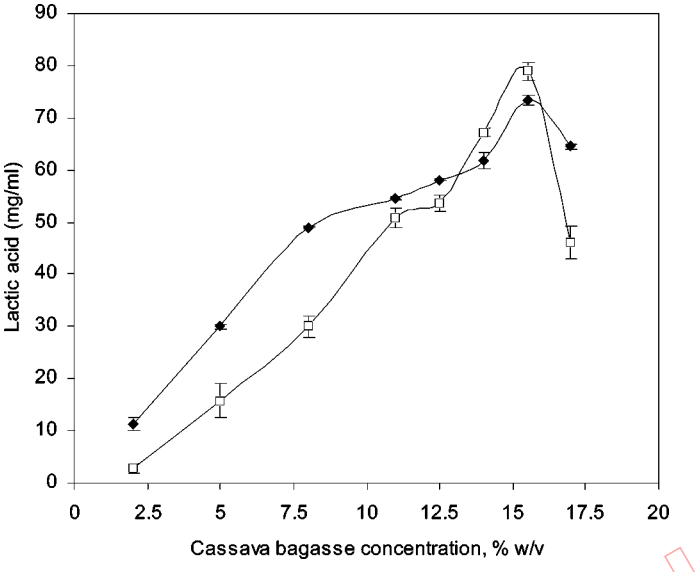

Fig. 1. Optimization of cassava bagasse concentration for simultaneous saccharification and fermentation; *L. delbrueckii* (◆), *L. casei* (□).

inoculum. However, we found an initial level of 15. Five percent (w / v) of starchy material in a shake-flask fermentation is really the best level of the raw material to start the process. When we made the hydrolysate by enzymatic treatment from cassava bagasse and used the hydrolysate for fermentation, it was found more than 7.5% (w / v) of starch cannot be used. We observed a kind of feedback inhibition as a result of higher glucose level in the hydrolysate (data not shown). Because of the controlled release of sugars and its utilization in simultaneous saccharification and fermentation, a higher level of initial raw material can be used.

Hujanen and Linko *(18)* have reported that 90% yield (121 g / L lactic acid from 130 g / L glucose supplemented with 20 g / L yeast extract by 15% inoculum of *L. casei*. The low lactic acid production rate was as a result of the high glucose concentration and production temperature. In this present study, 2% (v / v) inoculum is needed for the *L. casei* NCIMB 3254. However, 2.5% (v / v) inoculum was the optimum for *L. delbrueckii* NCIM 2025 (Fig. 2). Fig. 2

Lactic acid bacteria generally have complex nutrient requirements for the growth and fermentation *(5,19)*. In case of *L. delbrueckii* NCIM 2025 and *L. casei* NCIMB 3254, optimum yeast extract concentration was 0.5% (w / v) for the maximum conversion (Fig. 3). Lower concentrations had a negative effect on the productivity. This observation is in good agreement with the report on lactic acid production from date juice using *L. casei (20)*. There was no influence of yeast extract in higher concentrations. Fig. 3

Similarly, as shown in Fig. 3, an optimum of 0.5% (w / v) of NH_4Cl was supplemented in the production medium along with 0.5% (w / v) of yeast extract, which serves as a readily available nitrogen source for the organism.

Job: ABAB 134
Chapter: Nampoothiri (05-0090)
Pub Date: 2006
Operator: KB
Date: 5/06
Revision: 1st galleys

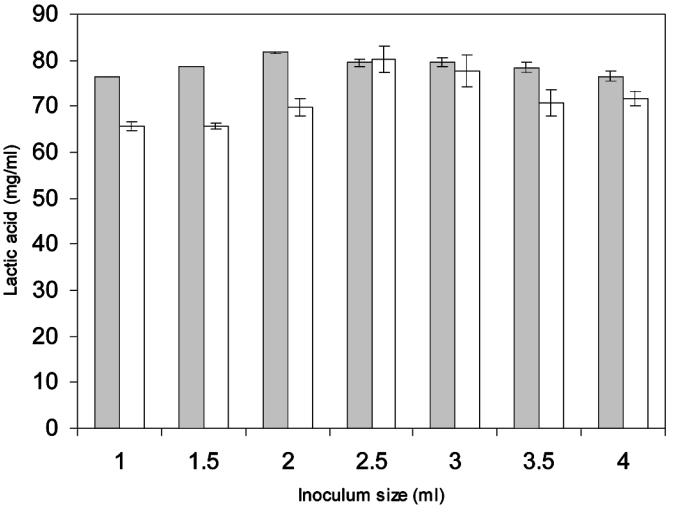

Fig. 2. Optimization of inoculum size; *L. delbrueckii* (□), *L. casei* (■) for the lactic acid production.

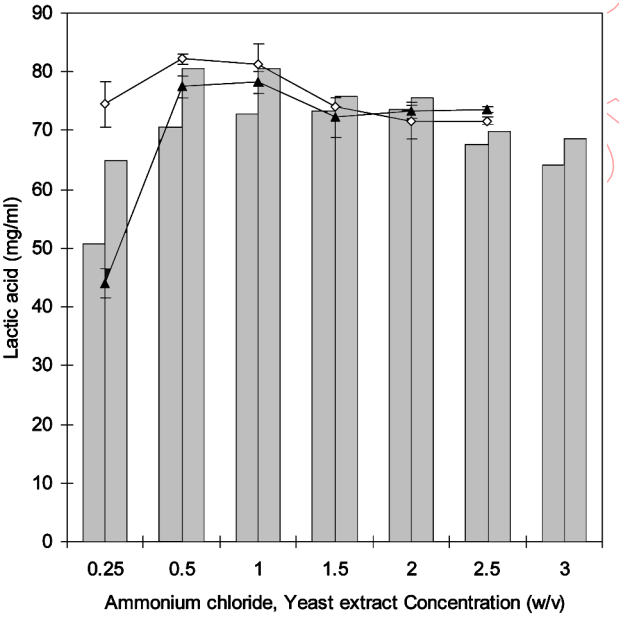

Fig. 3. Optimization of yeast extract concentration; *L. delbrueckii* (▲), *L. casei* (◇) and ammonium chloride concentration; *L. delbrueckii* (■), *L. casei* (□) for the production of lactic acid.

A time course study showed that for both *L. delbrueckii* and for *L. casei* the lactate production reached a plateau after 60 h. Further increase in fermentation time did not enhance the yield. As it is shown in Fig. 4, the

Fig. 4

Job: ABAB 134
Chapter: Nampoothiri (05-0090)
Pub Date: 2006
Operator: KB
Date: 5/06
Revision: 1st galleys

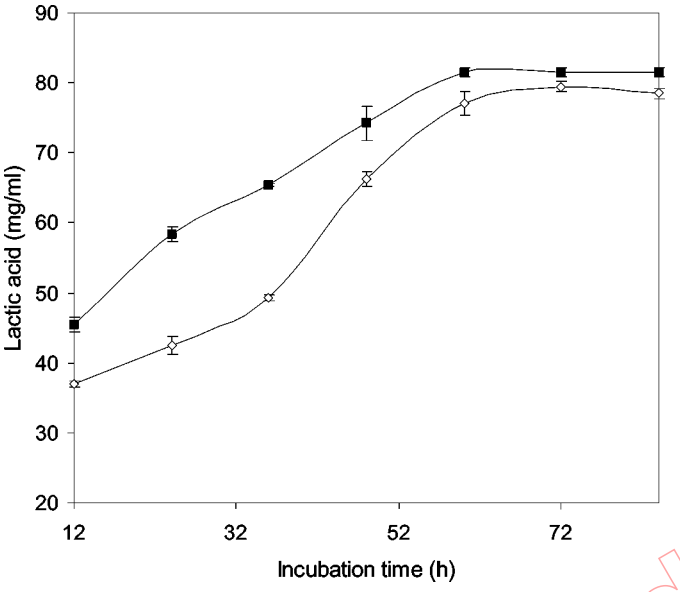

Fig. 4. Optimization of incubation time during the cultivation of lactobacilli; *L. delbrueckii* (◇), *L. casei* (■).

maximum production of lactic acid using *L. delbrueckii* was 79.5 mg / mL and it was 81.5 mg / mL for *L. casei.*

Lactic acid bacteria have a wide range of temperature optimum for lactic acid production. Linko and Javanainen *(7)* showed that with *L. casei* an optimum conversion of 87–98% was occurred at 37°C and it was 77–82% at 41°C. Anuradha et al. *(2)*, conducted experiment with *L. delbrueckii* at a temperature of 40–50°C and obtained a 70% conversion of starch. In this study, maximum production was noted when both the organisms were incubated at 37°C (80.6 mg / mL in the case of *L. casei* and 74.4 mg / mL for *L. delbrueckii*). At all temperatures *L. casei* had the better yield than *L. delbrueckii*, except at 44°C (Fig. 5).

Fig. 5

Fig. 6

Interestingly, as shown in the Fig. 6, we observed that the presence $MnCl_2$ in lower concentration level (0.01% [w / v]) enhanced the lactic acid production in the case of *L. casei.* However, in higher concentrations, it had an inhibitory effect and the production is less than that of the control one. In the case of *L. delbrueckii*, the $MnCl_2$ did not show any positive effect. In comparison to our observation there are reports of enhanced lactic acid production in presence of $MnCl_2$ with *L. casei (21).*

Calcium carbonate plays a crucial role in the lactic acid fermentation as it buffers the medium. During the production of lactic acid, it is converted to calcium lactate and maintains the pH. The presence of 2.5–4% (w / v) of calcium carbonate in the production medium was found to be good for lactic acid production in both the cases. In the control, the accumulation of lactic acid is inhibited because of the lowering of pH, which adversely affected the bacterial growth. After 60-h fermentation under regulated

Job: ABAB 134
Chapter: Nampoothiri (05-0090)
Pub Date: 2006
Operator: KB
Date: 5/06
Revision: 1st galleys

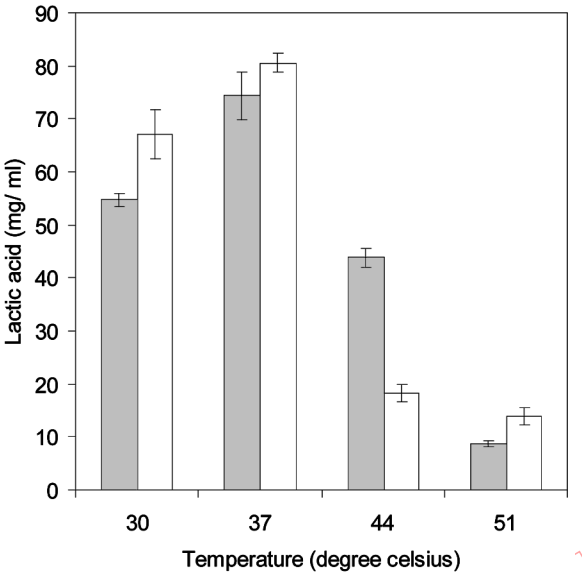

Fig. 5. Optimization of fermentation temperature; *L. delbrueckii* (■), *L. casei* (□).

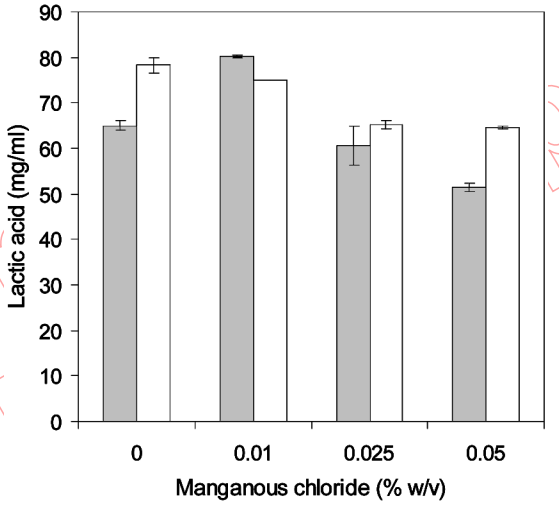

Fig. 6. Optimization of $MnCl_2$; *L. delbrueckii* (□), *L. casei* (■).

pH conditions, *L. casei* produced 83.8 mg / mL of lactic acid with a conversion (sugar to lactic acid) of 96.3% and for *L. delbrueckii* it was 81.9 mg / mL with a conversion of 94.1%. The rate of productivity was calculated accordingly. Data are shown in the Fig. 7.

Fig. 7

Woiciechowski et al. *(13)* studied the hydrolysis of cassava bagasse starch by acid and enzymatic hydrolysis and reported that both the methods were quite efficient depending on the percentage of hydrolysis, time, and cost of the chemicals and energy consumption. According to them, the

Job: ABAB 134
Chapter: Nampoothiri (05-0090)
Pub Date: 2006
Operator: KB
Date: 5/06
Revision: 1st galleys

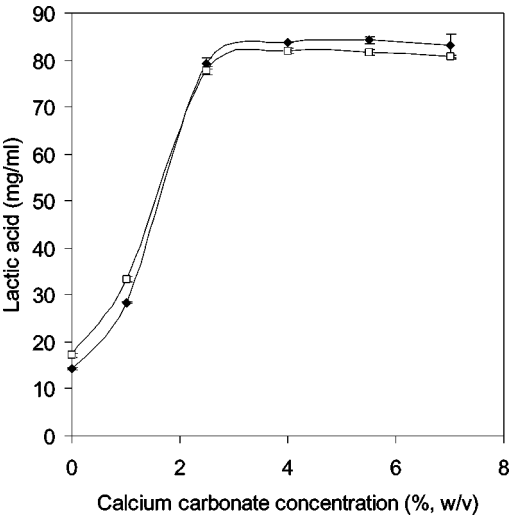

Fig. 7. Optimization of $CaCO_3$ concentration; *L. delbrueckii* (□), *L. casei* (◆).

enzymatic hydrolysis of 150 kg cassava bagasse itself is required around $2471. The advantage of simultaneous saccharification is that it avoids the separate hydrolysis step and reduces the cost of energy consumption for the liquefaction and saccharification Hence it is cost effective and time saving process for the lactic acid production.

Conclusion

A cost-effective and eco-friendly method was adopted with the valuable addition of cassava bagasse, which contains nearly 52% entrapped starch. A single step conversion of cassava starch present in the bagasse to L-lactic acid is demonstrated using two lactobacilli. The tedious preparation of separate starch hydrolysate either by the enzymatic or chemical methods is avoided with the simultaneous saccharification and fermentation process. Also, a comparatively large amount of initial raw material can be used because there is a controlled release of sugar and its immediate use. Hence, the uncompetitive inhibition by the substrate generally observed while using starch hydrolysate because of high the concentration of sugar is avoided.

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Job: ABAB 134
Chapter: Nampoothiri (05-0090)
Pub Date: 2006
Operator: KB
Date: 5/06
Revision: 1st galleys

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