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STUDIES ON FACTORS AFFECTING ETHANOL PRODUCTION FROM CELLULOSIC WASTES BY A MESOPHILIC *CLOSTRIDIUM* SPECIES

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A mesophilic cellulolytic *Clostridium* sp was isolated under anaerobic conditions on cellulose agar medium. The bacterium fermented variety of crystalline and native cellulose. Ethanol was found as one of the major fermentation products. The bacterium produced 0.21 g of ethanol per gram of cellulose consumed. Maximum ethanol production was found at pH 7.5 and temperature 35°C. Presence of carbondioxide in the culture vials was found to inhibit the ethanol production by 40%. pH had little effect on ethanol production at low substrate concentration. Among the crystalline and pure cellulosic materials tested ethanol production was maximum during fermentation of whatman filter paper.

Key Words: *Clostridium* sp, lignocellulosic wastes, ethanol production, mesophilic condition.

Cellulose is a linear polymer made up of glucose subunits linked by ß, 1-4 glycosidic bonds. It is estimated that global synthetic rate of cellulose is approximately $4x10^7$ tons per year (Singh & Hayashi, 1995). Energy is obtained from biomass by direct combustion or by heat treatment or by microbial action. Among the microbial conversion of biomass to fuels, production of ethanol has great potential and is one of the best ecofriendly technologies for biomass conversion to energy.

Cellulolytic bacteria ferment cellulosic biomass to ethanol. (Lovitt *et al.*, 1988, Sairam *et al.*, 1991, Lynd *et al.*, 1991, Sudha Rani *et al.*, 1996). They hydrolyse both cellulose and hemicellulose substrate to their oligodextrins and monomeric components. *Clostridium thermocellum* is able to ferment cellulose and cellobiose producing ethanol as the major fermentation product, (Nochur *et al.*, 1992). In the present study production of ethanol by a mesophilic cellulolytic bacterium from lignocellulosic wastes and the factors affecting the ethanol production were studied.

MATERIALS AND METHODS

Mesophilic *Clostridium* sp used in this study has been isolated under anaerobic conditions from sewage mud following the technique of Hungate (1969) on cellulose medium containing (g/l) K_2HPO_4-2.21, KH_2PO_4-1.5, $MgSO_4$-0.8, $CaCl_2$-0.02, yeast extract-0.5, cellulose powder-10.0. The culture was maintained on the same medium with 0.4% cellulose. This bacterium produced ethanol as major fermentation product, besides the production of acetic acid and butyric acid (Vijaya & Dev, 1996).

All the experiments were conducted in serum vials of 120 ml capacity containing 20 ml of the prereduced medium supplemented with 10% finely graded paddy straw as substrate after delignification with 8% NaOH. The medium was sterilized by autoclaving at 121°C for 30 min. A 5% inoculum grown on 0.4% cellulose for 72 hours (OD_{520} of about 0.7) was used as inoculum. All the incubations were carried out at 35°C ± without shaking for 10 days.

To determine the effect of pH on ethanol production the pH of the medium was adjusted to 5.0, 6.0, 6.5, 7.0, 8.0 and 8.5 with sterile anaerobic 8M NaOH after sterilization. Inoculated the vials, the effect of temperature on ethanol production was studied by incubating the inoculated serum vials at various temperatures ranging from 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C. To know the effect of substrate concentration, the finely graded paddy straw was used at concentration of 10 g/l, 20 g/1,30 g/1, 40 g/1 and 50 g/l. The effect of substrate concentration on ethanol production was studied under two different conditions. The pH for one set was initially adjusted to 7.5 and was not maintained through out the experiment though there was a drop of pH on 5th day of experiment due to production of acetic and butyric acids. For another set of experiment the pH of vials was

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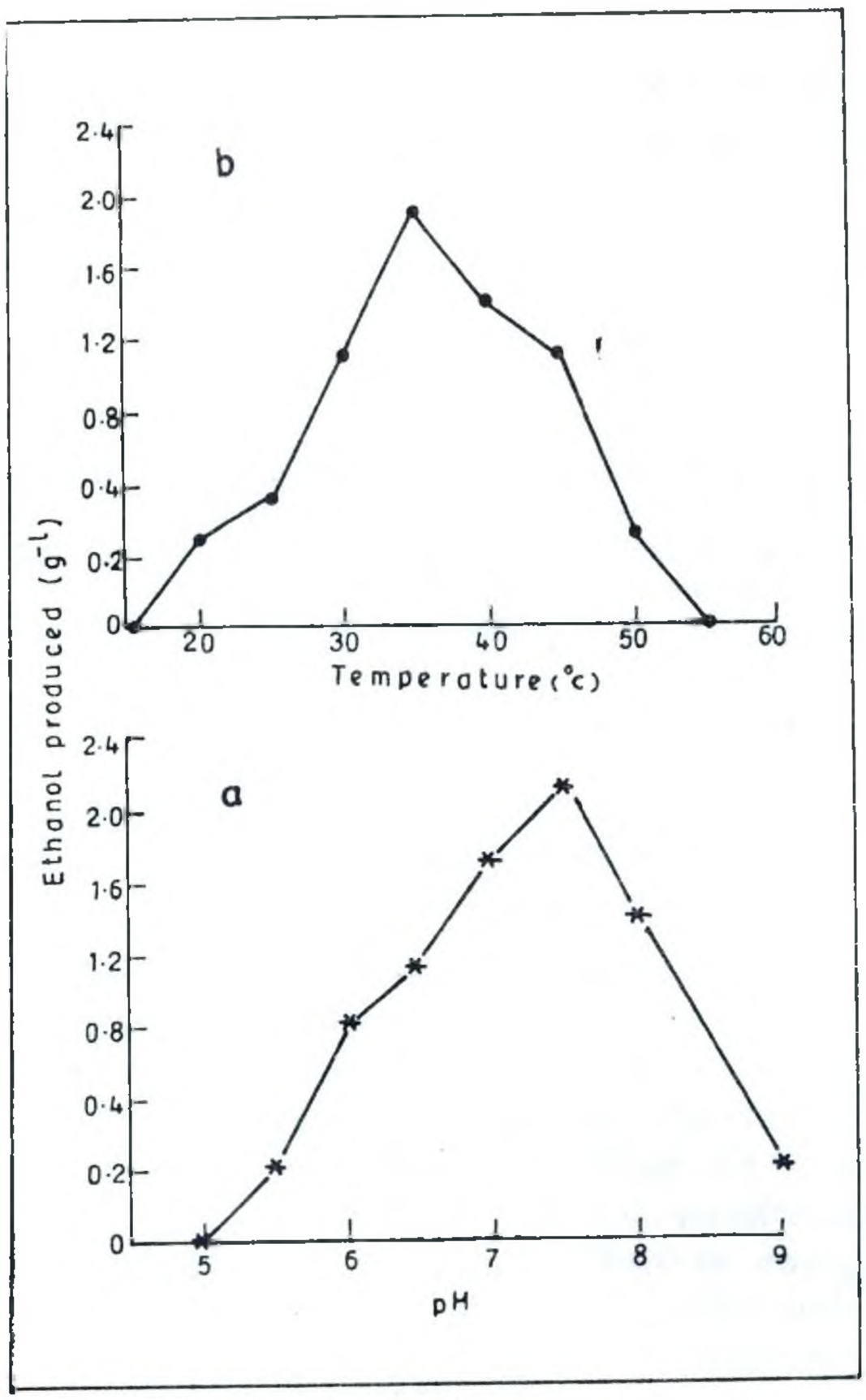

Figure 1. Effect of pH (a) and Temperature (b) on ethannol production.

maintained at 7.5 through out the experiment by periodic injection of 8M NaOH at every 24 hours. The head space gas used in all the above experiments was 100% N_2. To know the effect of head space gas on ethanol production N_2 was replaced by either 100% H_2, or 80% H_2 + 20% CO_2 atmosphere and incubated. Various crystalline and pure cellulosic substrates such as filter paper, tissue paper, avicel, native cotton and muslin cloth were used at concentration of 10% and the amount of ethanol produced from each substrate was calculated separately.

After incubation for 10 days the vials were chilled at 4°C to avoid the evaporation of ethanol during desealing of the vials. 10 ml of the culture fluid was taken in centrifuge tube and centrifuged at 4°C at 15,000 rpm for 30 min. The supernatant was collected and acidified with 1ml of 2N phosphoric acid

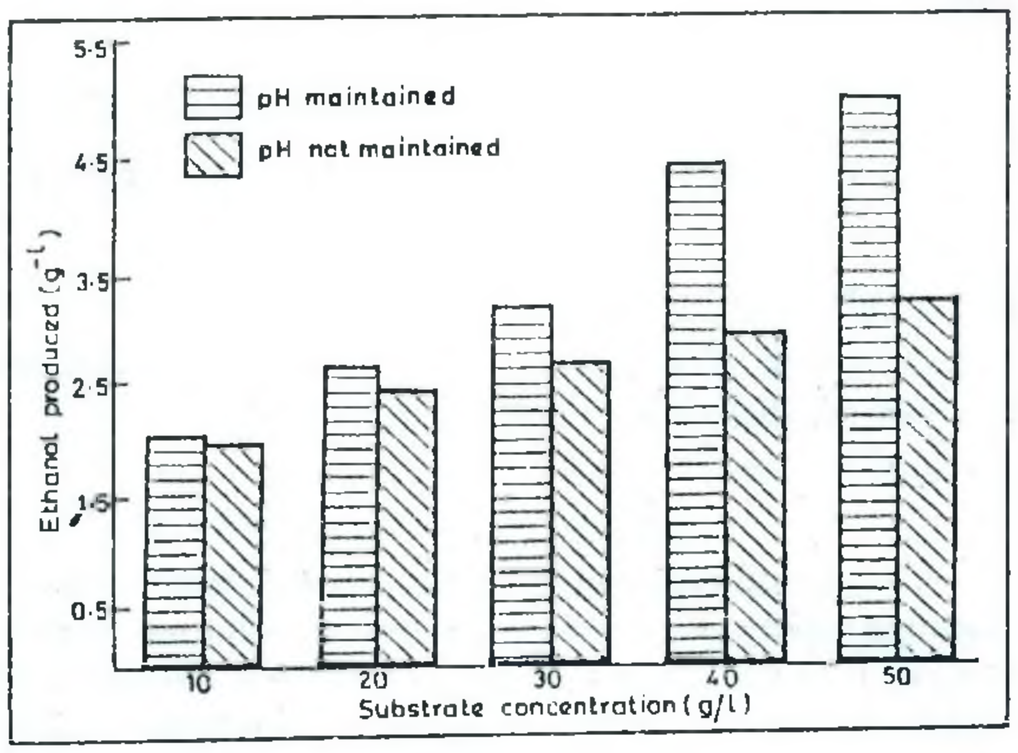

Figure 2. Effect of substrate concentration on ethanol production.

and 2 µl of this was injected into gas chromatography equipped with FID (Flame Ionization Detector) and a stainless steel column packed with chromosorb 101, 80-100 mesh. The following parameters were choosen for analysis. Oven temperature 160°C, injection temperature 170°C, carrier gas N_2, flow rate 20ml/min. All the values presented are the average of three replicates of two independent experiments.

RESULTS AND DISCUSSION

The mesophilic cellulolytic *Clostridium* sp which was isolated from sewage mud under anaerobic condition was gram positive straight rod with terminal spores and motile by peritrichous flagella. Sporulation was appreciable at >0.2% substrate concentration and was less frequent at higher concentrations. This bacterium fermented various crystalline and native cellulose. The organism produced acetic acid, butyric acid, ethanol and gaseslike hydrogen during fermentation of cellulose. Ethanol was one of the major fermentation product. The bacterium produced 0.22 g of ethanol per gram of cellulose consumed. (Vijaya, 1993).

In the present study it was found that the pH below and above 7.5 inhibited the production of ethanol (Fig. 1a). Maximum ethanol production was found at temperature 35°C, with the increase in temperature there was a gradual decrease in the yield of ethanol (Fig. 1b). Production of ethanol is found associated with enzymes alcohol dehydrogenase and ferredoxin NAD reductase activity. Increase in pH or temperature inhibit the activity of these enzymes (Lamed and Zeikus, 1980, Madden *et al.*, 1983) which afffects the ethanol production.

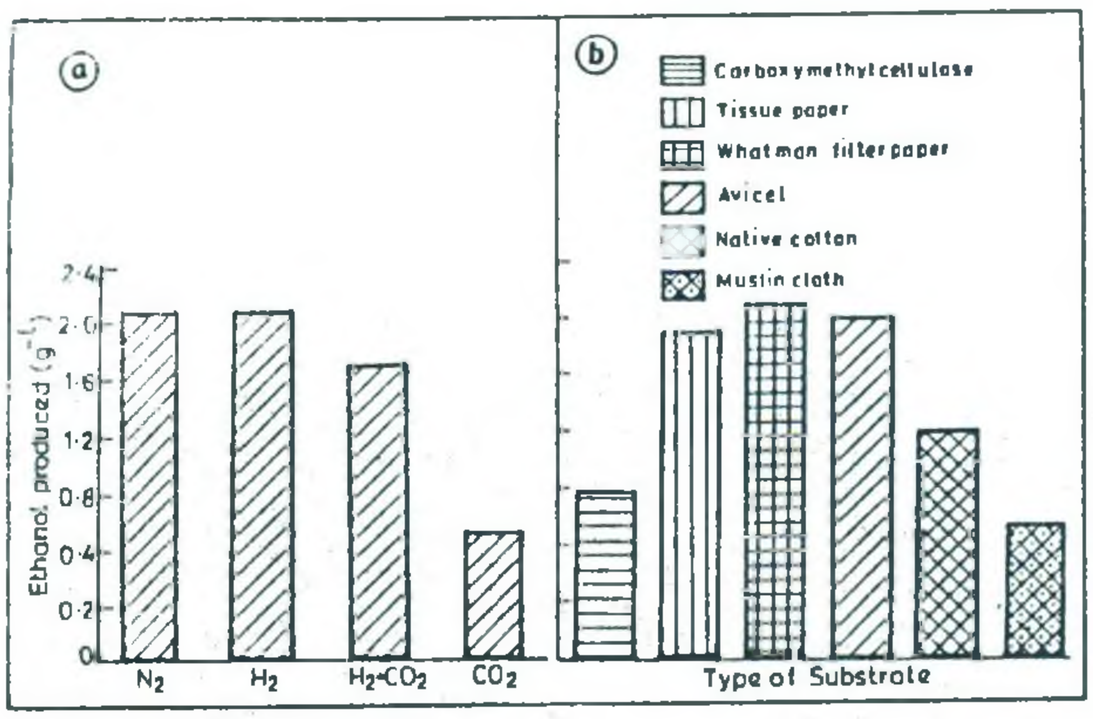

Figure 3. Effect of different headspace gases (a) and type of substrate (b) on ethanol production.

At the substrate concentration of 10 g/1 and 20 g/1, pH had little effect on ethanol production (Fig. 2) and at concentration above 30 g/1 the amount of ethanol produced was lowered. These observations support findings of Sairam and Seenayya (1989). The *Clostridium* sp showed differential response to the head space gas composition in the serum vials. Ethanol production was not affected by 100% H_2, or 80% H_2 + 20% CO_2 atmosphere, whereas 40% decrease in ethanol production was found when 100% N_2 was replaced with 100% CO_2 (Fig. 3a). Earlier Lamed and Zeikus (1980) reported the positive influence of H_2 on ferredoxin NAD reductase activity which influence the ethanol production. The low yield of ethanol in 100% CO_2 may be due to specific regulatory effect on these enzymes. This bacterium could able to ferment a variety of crystalline and pure cellulosic materials. The amount of ethanol produced was maximum when whatman No.1 filter paper was used as substrate, followed by avicel, tissue paper, cotton and carboxymethyl cellulose and muslin cloth (Fig. 3 b). The rate of degradation was found dependent on the complexity of the substrate (Khan and Murray, 1982). Muslin cloth having high degree of polymerization between 8000 to 10,000 degraded comparatively less with low ethanol yield than other fairly refined cellulosic materials.

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