EXPERIMENT-2

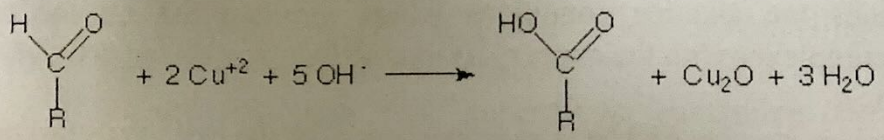
Aim: To produce hydrolytic enzymes (cellulase) in shake flask and confirm using Benedict's test.

Introduction/Principle:

Hydrolytic enzymes include cellulase, cellobiase, xylanase and amylase for converting carbohydrates into sugars, protease for hydrolysing proteins into amino acids and lipase for degrading lipids into glycerol and long chain fatty acids (LCFA). The hydrolytic enzyme, "cellulase", is an enzymatic complex composed of endo-1,4-β-d-glucanases or endoglucanases, exo-1,4-β-d-glucanases or cellobiohydrolases and 1,4-β-d-glucosidases, which act on cellulose to produce glucose. Cellulose is one of the most abundant biodegradable materials on the Earth which can be produced by many organisms, including bacteria and vascular plants. As a kind of important industrial enzyme, cellulase has been widely used in the feed industry, alcoholic fermentation, fruit juice and other fields. The utilization of cellulase in animal feed has been reported widely.

Bacillus is a kind of probiotics that can secrete high activity of protease, lipase, amylase and cellulase. *Bacillus amyloliquefaciens* is an important potential probiotic strain that has been found to secrete cellulase and applied to many types of mammalian feed for improving their intestinal microenvironment (Miao Ye et al., *2017*). This same host organism will be used for harvesting cellulose in this experiment.

Benedict's Test is used to test for simple carbohydrates. The Benedict's test identifies reducing sugars (monosaccharide's and some disaccharides), which have free ketone or aldehyde functional groups.
Some sugars such as glucose are called reducing sugars because they are capable of transferring hydrogens (electrons) to other compounds, a process called reduction. When reducing sugars are mixed with Benedict's reagent and heated, a reduction reaction causes the Benedicts reagent to change color. The color varies from green to dark red (brick) or rusty-brown, depending on the amount of and type of sugar.

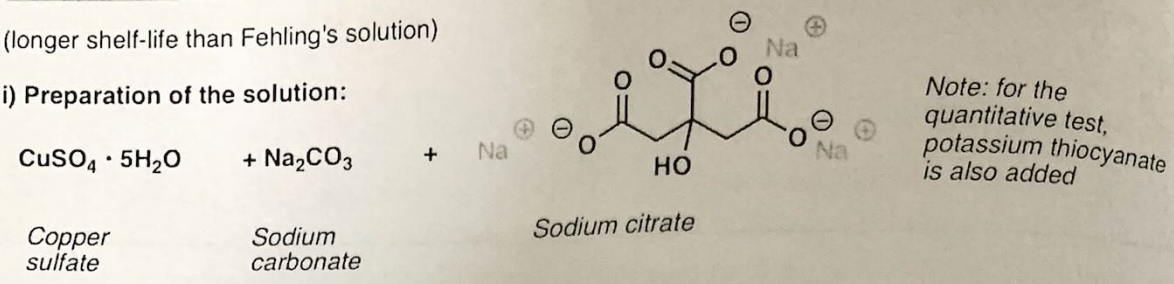


Benedict's quantitative reagent contains potassium thiocyanate and is used to determine how much reducing sugar is present. This solution forms a copper thiocyanate precipitate which is white and can be used in a titration. The titration should be repeated with 1% glucose solution instead of the sample for calibration (standard).

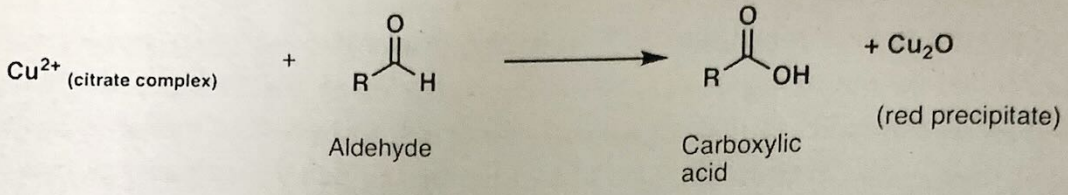
Benedict's Solution

(longer shelf-life than Fehling's solution)

i) Preparation of the solution:



ii) Use in oxidation reaction



Principle of Benedict's Test

When Benedict's solution and simple carbohydrates are heated together, the solution changes to orange red/ brick red colour. This reaction is caused by the reducing property of simple carbohydrates. The copper (II) ions in the Benedict's solution are reduced to Copper (I) ions, which causes the color change.

The red copper(I) oxide formed is insoluble in water and is precipitated out of solution. This accounts for the precipitate formed. As the concentration of reducing sugar increases, the nearer the final colour is to brick-red and the greater the precipitate formed. Sometimes a brick red solid, copper oxide, precipitates out of the solution and collects at the bottom of the test tube.

Sodium carbonate provides the alkaline conditions which are required for the redox reaction. **Sodium citrate** complexes with the copper (II) ions so that they do not deteriorate to copper(I) ions during storage.

Complex carbohydrates such as starches DO NOT react positive with the Benedict's test unless they are broken down through heating or digestion (try chewing crackers and then doing the test). Table sugar (disaccharide) is a non-reducing sugar and does also not react with the iodine or with the Benedict Reagent. Sugar needs to be decomposed into its components glucose and fructose then the glucose test would be positive but the starch test would still be negative.

Composition and Preparation of Benedict's Solution

Benedict's solution is a deep-blue alkaline solution used to test for the presence of the aldehyde functional group, – CHO.

Anhydrous sodium carbonate = 100 gm
Sodium citrate – 173 gm
Copper(II) sulfate pentahydrate = 17.3 gm

One litre of Benedict's solution can be prepared from 100 g of anhydrous sodium carbonate, 173 g of sodium citrate and 17.3 g of copper(II) sulfate pentahydrate.

List of reagents and instruments:

A. Organism

E. coli BL - 21 (DE3) pLysS cells harboring recombinant plasmids of BaGH5 - WT or BaGH5 - UV2

B. Equipment

Erlenmeyer flask, shaker incubator, centrifuge, microcentrifuge tubes, UV-Visible spectrophotometer.

C. Reagent

Benedict's reagent

D. Media composition

The medium composition, used for *E. coli* BL - 21 (DE3) pLysS cells production was LB broth (Luria Bertani broth)

Procedure:

1. Medium was prepared according to given composition and autoclaved. After this the antibiotic kanamycin (50 µg/ml) was added to the medium.
2. A 250 mL Erlenmeyer flask containing 100 mL of medium was inoculated with 7% (v/v) culture
3. The cells were grown at 37°C, and 180 rpm up to midexponential phase to cell absorbance at 600 nm (A600) ~0.6. A
4. 1.0mM final concentration of isopropyl - 1 - thio - β - D - galactopyranoside (IPTG) was added, and it was further incubated at 24°C, 180 rpm for 18 hr for induction of protein expression.
5. The cells were then centrifuged at 8,000g at 4°C for 10 min. The cell pellet(s) were resuspended in 4ml of 20mM sodium phosphate buffer, pH 7.0
6. After this, the cell suspension was sonicated on ice for 15min by using 5 s on/5 s off pulse and 33% amplitude.
7. Now, the sonicated cell suspension was again centrifuged at 13,000g at 4°C for 50 min. and the supernatant was collected.
8. Finally, the cell free supernatant containing the enzyme was analysed for endoglucanase (CMCase) activity using Benedict test.

Note: In case of mutant culture test tubes are incubated in dark (use foil).

Benedict's test procedure:

1. Three test tubes were taken marked as Positive control (containing only glucose), Negative control (containing only CMC) and CMC+supernatant (containing enzyme) - 2ml each.
2. The three test tubes were incubated for two hours at room 65°C temperature.
3. After incubation, 2ml of Benedict's reagent was added to each test tube.
4. The test tubes were kept in boiling water bath for 5/10 minutes and the change in colour/precipitate formation was observed.

Result Interpretation of Benedict's Test

If the color upon boiling is changed into green, then there would be 0.1 to 0.5 percent sugar in solution.
If it changes color to yellow, then 0.5 to 1 percent sugar is present.
If it changes to orange, then it means that 1 to 1.5 percent sugar is present.
If color changes to red,then 1.5 to 2.0 percent sugar is present.
And if color changes to brick red,it means that more than 2 percent sugar is present in solution.

Positive Benedict's Test: Formation of a reddish precipitate within three minutes. Reducing sugars present. Example: Glucose
Negative Benedict's Test: No color change (Remains Blue). Reducing sugars absent.

Results Observed from Experiment:

Cellulase enzyme was isolated successfully after 48 hours of culture period.

After performing the Benedict's test, the following observations were made:

1. The first test tube (positive control) developed red-brick colour precipitate due to the presence of only glucose.
2. The second test tube (negative control) developed blue colour due to the presence of only CMC and absence of glucose (reducing sugar).
3. The third test tube, colour slightly changed from blue to red, which confirmed the presence of the cellulase enzyme in the supernatant.

Conclusion

The hydrolytic enzyme cellulase was successfully isolated from the culture of *Bacillus amyloliquefaciens* SS35 and confirmed using Benedict's test. The colour change from blue to red through Benedict's test confirmed the presence of the cellulase enzyme from the supernatant harvested from the culture.

References

1. Singh, Shuchi, Vijayanand S. Moholkar, and Arun Goyal. "Optimization of carboxymethylcellulase production from Bacillus amyloliquefaciens SS35." *3 Biotech* 4, no. 4 (2014): 411-424.
2. Singh S, Kumar K, Nath P, Goyal A. Role of glycine 256 residue in improving the catalytic efficiency of mutant endoglucanase of family 5 glycoside hydrolase from Bacillus amyloliquefaciens SS35. Biotechnol Bioeng. 2020 Sep;117(9):2668-2682. doi: 10.1002/bit.27448. Epub 2020 Jun 30. PMID: 32484905.