Practical 1:Starch-Digesting Enzymes from Barley and Cellulose-Digesting Enzymes from Almonds

By: Edward Blake

ID Card Number: 171098M

University of Malta

Aim:

To determine whether germinating barley seeds contain amylase. To determine whether germinating almond seeds contain cellulase. To determine if lignin limits cellulase from breaking down cellulose and if lignin weakens this property under heat.

Introduction:

In any alcohol beverage industry, the requirement of monosaccharide is a must to allow for microbial fermenters, such as, yeast to easily breakdown the simple sugars into alcohol in an anaerobic environment. Within this practical, the studying of the process used in creating these simple sugars from barley shall take place. This process requires the usage of amylase enzyme that is produced by germinating barley seeds. Germinating barley seeds synthesize amylases during germination due to the fact that their endosperm (food storage) is composed of starch which needs to be broken down, Bilderback. (1971). As such, pre-soaking the seeds with water will allow the germination process to commence where one can then take full advantage and extract the amylase needed for the production of simple sugars. The presence of this amylase enzyme shall be proven by aqueous extraction of the amylase from germinated barley seeds via crushing and centrifugation. This aqueous extract will then be tested by adding it into varying corn starch concentrations with varying enzymatic extract quantities. This will allow for qualitatively determining the presence of glucose/maltodextrin and the absence of starch, which will both allow for the confirmation that the aqueous extract contains amylase. The absence of starch will be tested via the iodine test which will have a blue/purple colour if starch is present or will have a brown/yellow colour if no starch is present. Therefore, a hypothesis shall be stated that if the absence of starch is determined from a known quantity of corn starch, then there is amylase present in germinating barley seeds.

The creation of large quantities of ethanol requires staggering amounts of glucose. As such, within this day and age, the breakdown of the large glucose chains called cellulose takes place. Within the second experiment, the detection of cellulase found in almond seeds shall take place. The cellulase found in the almond seeds react on the glycosidic bonds found in cellulose, thereby breaking down the cellulose into simple sugars. As such, within this practical, one shall first detect the presence of cellulase in almond seeds via the presence of maltodextrin and glucose via Fehling's test, which turns reddish brown in the presence of simple sugars. Hence the hypothesis that if glucose is detected after adding the almond seed aqueous extract to the cellulose source, then cellulase is present in almond seeds. The major problem with the breakdown of cellulose into simple sugars is the presence of lignin which is a strong polymer which links cellulose chains together and is resistant to cellulase, therefore it will limit cellulase's breakdown of cellulose into glucose. As such, when exposing three sources of cellulose with different concentrations of lignin one will notice that the lower lignin concentrated source will produce more glucose molecules. Hence hypothesis, if lower concentrated lignin sources possess more glucose than higher lignin concentrated cellulose after addition to cellulase enzymes, then lignin limits cellulose break down. Furthermore, a paper by Reddy et al. (2017) had shown that lignin loses strength significantly when heated. As such, a hypothesis shall be stated that if there is a higher presence of glucose when cellulase is added to boiled wood, paper and cotton samples than that of identical room temperature cellulose/lignin sources then lignin reduces efficiency in preventing cellulase from breaking down cellulose.

Procedure:

For protocol refer to source below, by Buhagiar. (2021):
https://www.um.edu.mt/vle/pluginfile.php/1107613/mod_folder/content/0/Biotechnology%20II%20Practical%201%20-%20Carbohydrate%20Digesting%20Enzymes%20JB%20October%202021.pdf?forcedownload=1

Modifications to protocol:

Experiment 1:

- One shall pre-soak $20\ g$ of barley seeds with $10\ ml$ of dH_2O (not $20\ ml$ of dH_2O) and crush it into a smooth paste.
- One must repeat the test following addition of the barley's supernatant with corn starch every $15\ min$ not every $3\ min$.
- The reaction of the barley's supernatant with corn starch will only be performed at room temperature.

Table 1 have been modified as the following:

Group Number:	Volume of 1% and 0.1% (w/v) CS solution (µL):	Volume of enzymatic supernatant (µL):
1	400	600
2	300	700
3	200	800
4	600	400
5	500	500
6	600	400
7	500	500

Sources of Errors:

- When crushing the seeds there was the human error of uneven crushing, hence some groups' extract may be more concentrated than others.
- The surface area of the wood samples differed greatly from test to test, hence it is possible that more finely shaven wood chips will have a disproportionately higher glucose concentration than other wood samples.
- Different seeds contain different concentrations of amylase or cellulase, thus, there will be a disproportionate concentration of amylase and cellulase between groups.

Precautions:

- When mixing the corn starch in the deionized water, one must make sure no starch blobs are present so as to allow for full enzymatic reaction.
- The barley must be crushed into a smooth pasted so as to extract as much amylase as possible.
- Care was taken when handling phloroglucinol and only used within the fume hood as it can cause mild nausea on exposure, hence Shin et al., (2020).

Results:

Table 1:

Group Number	**Volume of Corn-Starch Solution (μl)**	**Volume of Enzymatic Extract (μl)**	**Completion Time (minutes)**	
			1% CS Solution	**0.1% CS solution**
1	400	600	45	Incomplete Reaction
2	300	700	30	0
3	200	800	45	15
4	600	400	60	60
5	500	500	45	45
6	600	400	Incomplete Reaction	45
7	500	500	Incomplete Reaction	60

Note: This table contains the time for complete hydrolyzation of the starch using the aqueous extract of the barley seeds.

Table 2:

Percentage of Enzymatic extract relative to original batch strength (%)	**Average Completion Time (minutes)**	
	1% CS Solution	**0.1% CS solution**
40	45	52.5
50	30	52.5
60	45	Incomplete Reaction
70	60	0
80	45	15

Note: The data used originates from *Table 1*.

Table 3:

Group Number	Substrate Used	Completion Time (minutes)		Fehling's Test		Lignin Test	
		20-25˚C (room temperature)	**40 ˚C**	**20-25˚C (room temperature)**	**40 ˚C**	**20-25˚C (room temperature)**	**40 ˚C**
1	Wood pulp	Incomplete reaction	Incomplete reaction	Negative	Negative	Negative	Negative
2	Wood pulp	Incomplete reaction	Incomplete reaction	Negative	Negative	Negative	Negative
3	Paper	Incomplete reaction	Incomplete reaction	Negative	Negative	Negative	Negative
4	Paper	Incomplete reaction	Incomplete reaction	Negative	Negative	Negative	Negative
5	Cotton wool	Incomplete reaction	Incomplete reaction	Negative	Negative	Negative	Negative
6	Cotton wool	Incomplete reaction	Incomplete reaction	Partial Positive	Partial Positive	Negative	Negative
7	Wood pulp	Incomplete reaction	Incomplete reaction	Negative	Negative	Negative	Negative

Note: This table contains the temperature effect of the hydrolysis of cellulase extract on various cellulose and lignin containing substrates.

Discussion:

When viewing *Table 2*, it can be seen that as the concentration of the original batch strength of the barleys aqueous extract increases the time at which all corn starch is hydrolyzed decreases. As such, this will support the hypothesis that amylase is present in the aqueous extract, hence is also present in the barley seeds. As stated by Silano et al. (2017), the amylase found within germinating barley seeds is beta-amylase which will hydrolyze the glycosidic bonds of the starch thereby reducing this polysaccharide into maltose and/or glucose. The maltose can also be subject to further hydrolysis by amylase into glucose.

It was found by Novaes et al. (2010), that wood contains approximately 25% lignin and 70% cellulose. While Chang et al. (2021) found that wood contains approximately 10% lignin. Due to the limited information about the wood used within this practical, an assumption was made that the wood contained approximately 17.5% lignin. Małachowska et al. (2021) found that paper contains 3% to 14% lignin, hence within this practical the assumption that the paper used was approximately 8.5% lignin was taken. It was found by Chang et al. (2021) that cotton contains less than 1% lignin. While Kang et al. (2012) found a percentage range of 0.43% to 1.29% lignin concentration. Hence an assumption was made that the cotton used within this practical contained 0.86% lignin. As such, as seen in *Table 3*, it was found that only cotton contained simple sugars while the other higher lignin containing substrates did not. As such, this supports the two hypothesis that the almond seed aqueous extract contains cellulase and that lignin limits cellulase breaking down cellulose into simple sugars. Furthermore, support for the hypothesis that lignin weakens due to heat thereby increasing the cellulase's rate of cellulose breakdown was not found. Since within *Table 3*, there was no differences between the temperature and 40 ℃ test. But a review by Brebu & Vasile. (2010) shows that lignin does in fact weaken due to heat hence will support the hypothesis that cellulase will be more effective on the substrates, but further tests are needed to prove this.

To conclude, germinating barley seeds contain amylase which can be explored within alcoholic beverage factory by making the cost of breaking down polysaccharides into simple sugars cheaper. The presence of cellulase was also found within almond seeds. The cellulase is an excellent enzyme that is able to covert useless cellulose into large quantities of useful glucose, which can be used for ethanol production (for example, the 70% ethanol used to sterilize laboratories).

Furthermore, lignin is a complex polymer that will limit the breakdown of cellulose by cellulase, as such, it can be weakened using heat for more efficient cellulose breakdown.

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