



Review Article

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RECENT ADVANCES AND METHODS FOR *IN-VITRO* EVALUATION OF ANTIDIABETIC ACTIVITY: A REVIEW

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ABSTRACT

The antidiabetic properties of synthetic or natural substances can be evaluated by *in-vitro* methods such as study of glucose uptake, effect on glycosylation of the hemoglobin and inhibition of alpha amylase, alpha glucosidase and sucrase enzymes. The inhibitory nature of a test compound on alpha amylase enzyme reflects the antidiabetic activity because of unavailability of glucose from gastrointestinal tract. Alpha-glucose inhibitor reduces the impact of glucose on blood sugar and hence alpha glycosidase inhibitors can be considered as oral antidiabetic drugs. Once a hemoglobin molecule is glycated, because of high blood glucose level, it remains that way. Hence the estimation of glycated hemoglobin is important tool in the antidiabetic screening procedure. The inhibition of such glucose uptake by yeast cell is an important tool for the evaluation of antidiabetic property. Glucose diffusion assay is a simple diffusion method to evaluate the glucose movement *in vitro* and is expressed in terms of glucose diffusion retardation index (GDR). *In vitro* technique like cell culture is one of the important methods to evaluate the activity. Level of insulin secretion in culture of HIT-T15 cells is also reported for evaluation of antidiabetic activity.

Keywords: Alpha amylase; alpha glucosidase; sucrose inhibitory activity; pharmacological screening

INTRODUCTION

Diabetes is a disease in which the body does not produce or properly use insulin, which results in elevated blood sugar levels, a condition classically called as diabetes. The body needs insulin to convert sugar, starch and other foods into energy. Major symptoms of this disease are polydipsia, polyuria, weight loss etc. Several antidiabetic drugs such as alpha glycosidase inhibitors, sulfonylurea and biguanides derivatives are available in market, which have different pharmacological actions. No single drug is available as complete remedy to this. Several side effects are produced by the existing drug. The mentioned drugs produce several side effects such as abdominal pain, weight gain, vomiting, diarrhea, headache, dark urine, fluid retention, allergic reaction, hypoglycemia etc. Researchers are focused to develop the safer and single remedy for diabetic disorder. To evaluate the antidiabetic activity of synthetic or natural compound (isolated from traditional plants) *in-vitro* and *in-vivo* methods are used.¹⁻⁶

Many foods are rich in carbohydrates which are major energy providing molecules. Examples of such food include cereals, fruits and vegetables. Products of carbohydrate digestion, which results from enzyme mediated reactions during the process of digestion, are easily absorbable from small intestine. While simple carbohydrates require little or no enzyme for digestion, complex carbohydrates require specific enzymes. The specific enzymes required for the digestion of later include salivary amylase, maltase and pancreatic amylase. Some enzymes are necessary for the conversion of disaccharides to monosaccharides. Enzymes such as sucrase enzyme convert sucrose to glucose and fructose while lactase breaks lactose or milk sugar into glucose and galactose. These monosaccharides are absorbed in the small intestine and enter the blood through the hepatic circulation. Hence the inhibition of such

carbohydrate digesting enzymes is an important tool in the evaluation of antidiabetic activity since carbohydrates are important source of sugar. Inhibition of such enzyme like alpha-glycosidase and alpha-amylase that decrease the post-prandial increase of blood glucose present a valuable measure of antidiabetic activity. Figure 1 shows a diagram of enzymatic metabolism of carbohydrate in lumen.

The antidiabetic properties of synthetic or natural substances can be evaluated by *in-vitro* methods such as study of glucose uptake, effect on glycosylation of the hemoglobin and inhibition of alpha-amylase and alpha-glucosidase enzymes. The present scenario of pharmacological screening involves testing of new chemical entities as extract from plant or any material (synthetic /semi-synthetic) in isolated preparations followed by tests in whole animals. Rats, mice and sometimes higher animals like monkeys and dogs are used. Most drugs in use nowadays at therapy have been developed with these methods. Non-animal alternatives are advisable wherever necessary. The challenge for pharmacologist always will be to correlate *in-vitro* data with *in-vivo* findings. A key goal in pharmacological screening is a good understanding of the *in-vitro* performance. The current review focuses the various *in-vitro* methods for the evaluation of antidiabetic activity. *In-vitro* methods can play a very important role in the screening of antidiabetic activity of drugs and avoid or restrict animal use.

Screening of antidiabetic activity by using carbohydrate digesting enzymes

Alpha amylase inhibition

Alpha amylase enzyme is responsible for the hydrolysis of alpha bond linked polysaccharides such as starch and glycogen to disaccharides. Hence the inhibitory nature of the test compound reflects the antidiabetic activity because of unavailability of

glucose from gastrointestinal tract. Figure 2 shows a diagrammatic representation of mechanism of action of α -amylase. Alpha amylase inhibitors bind on alpha bonds on polysaccharides and prevent the conversion of polysaccharides to disaccharides and monosaccharides.^{7,8} The method involves mixing the sample with starch solution in beaker, adding alpha amylase to this mixture, stirring vigorously and finally incubating at 37°C for one hour. After incubation, 0.1 M sodium hydroxide is added to terminate the enzyme activity. The mixture is then centrifuged for 15 min and the glucose content in the superficial layer is determined by measuring the absorbance of coloured solution at 565 nm. The percentage inhibition is calculated using the formula:

$$\text{Percentage inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

Alpha glucosidase inhibitory activity

Alpha-glucose inhibitor reduces the impact of glucose on blood sugar. Hence alpha glycosidase inhibitors are employed as oral antidiabetic drugs. Alpha-glucosidase inhibitors are saccharides that act as competitive inhibitors of carbohydrate digesting enzymes, specifically alpha glucosidase enzyme in the brush border of the small intestines. The membrane-bound intestinal alpha-glucosidase hydrolyze oligosaccharides, trisaccharides and disaccharides to glucose and other monosaccharides in the small intestine.

In this method, different concentration of sample solution is incubated with 10 milliliters of enzyme solution for 10 minutes at 37°C. Maleate buffer pH 6.0 solution is used to make the volume. The enzyme reaction is started by adding solution of p-nitrophenyl alpha D-glucopyranoside and further incubated 37°C for 30 min. The reaction is terminated by treating the mixture in boiling water bath for 5 min. After the addition of 0.1M disodium hydrogen phosphate solution the absorbance of liberated p-nitrophenol is read at 400 nm.

Sucrase inhibitory activity

A bifunctional enzyme, sucrase-isomaltase catalyze hydrolysis of sucrose and isomaltose. The effect of sample on sucrose activity is assayed according to the method of Honda and Hara. In this method, different concentration of sample solution is incubated with ten milliliters of enzyme solution for 10 minutes at 37°C. Maleate buffer pH 6.0 solution is used to make the volume up to 210 μ L. The enzyme reaction is started by adding 100 μ L sucrose solution (60 mM) and incubating for 30 min at room temperature. To terminate the reaction, 200 μ L of 3,5-dinitrosalicylic acid reagent is added and the mixture is placed in a boiling water for five minutes. The absorbance of the solution is read at 540 nm. The percentage of inhibitory activities is calculated by using formula:

$$\text{Percentage inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

Evaluation of hemoglobin glycosylation

Glycation of protein frequently occurs, but in the case of hemoglobin a non-enzymatic pathway occurs between glucose and the N end of beta chain. Hemoglobin A_{1c} was first separated from other forms of hemoglobin using chromatographic column by color. When blood glucose levels are high, glucose molecules attach to the hemoglobin in red blood cells. The HbA1c level is

proportional to average blood glucose concentration over the previous 4 weeks to 3 months. Once a hemoglobin molecule is glycated it remains that way. Hence the estimation of glycated hemoglobin is important tool in the antidiabetic screening procedure. Figure 3 shows a diagram of hemoglobin glycosylation.

In this method, blood from healthy volunteers is placed into a bottle containing anticoagulant. Then blood is mixed with sodium chloride and one volume of carbon tetrachloride for the preparation of hemolsate according to hypotonic principle. The resulting hemolsate may contain debris, which is removed by centrifugation at higher rpm for 15 minutes at room temperature. The hemoglobin rich fraction is concentrated in the upper layer. One milliliter of hemoglobin fraction is transferred to test tube containing different concentration of glucose solution. Gentamycin (0.02%) and different concentration of test solution are added to the above mixture. All these solutions should be prepared in phosphate buffer. The reaction mixture is kept under incubation for 72 hours at 25°C. At different interval of incubation, the glycated hemoglobin is determined by spectrophotometry. The percentage of inhibition can be:

$$\text{Percentage inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

Glucose uptake by yeast cells

The characteristic of the sugar transport system in yeast have been receiving renewed attention in several laboratories.⁹ All yeasts presently known are able to utilize one or more sugars as their principal source of carbon and energy. The yeast converts this sugar to ethanol. The inhibition of such glucose uptake by yeast cell is an important tool for the evaluation of antidiabetic property of either synthetic or traditional plant based compounds. Figure 4 shows a diagrammatic representation of glucose uptake by yeast cell. Here one milliliter of glucose solution (at various concentrations such as 5, 10, 10 and 25 mM) is added to the test solution and incubated for ten minutes at 37°C. The yeast suspension is repeatedly washed with distilled water until the supernatant fluid is clear after centrifugation. A 10% v/v suspension is prepared with the supernatant fluid. The yeast suspension is placed to the mixture, vortexed and further incubated at 37°C for 60 minutes. After incubation, the mixture is centrifuged for 5 minutes and the glucose in the supernatant layer is estimated. An existing antidiabetic drug is taken as standard. The percentage increase in glucose uptake by yeast cells is calculated using the formula:

$$\text{Percentage inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

Model to study insulin secretion from beta cells of the pancreas

The HEPG2ins/g cells are used to study insulin secretion from beta cells of the pancreas. This cell line is also called as betacyte, which is genetically engineered insulin-secretary human liver cells line.¹⁰⁻¹² The insulin secretary HIT cell line is developed by isolating pancreatic islets from hamster¹³ dispersing the islets into single cells, transforming the cell isolates with the Simian virus 40(SV40), and cloning out the insulin secretary cell lines. Experiments for assessment of glucose transport activity in HTT cells and western blot analysis for GLUT2 in these cells after incubation with glibenclamide and troglitazone were performed by Masuda et al.¹⁴

Model based on muscle as an insulin target tissue

The effect of insulin on glucose uptake in the soleus muscle of rats during hemorrhagic shock has been studied. Adipocytes are incubated with D(U-14C) glucose (0.2 mM final concentration) for 20 min. Cells are separated from the medium by centrifugation on silicon oil, removed and counted for radioactivity. The assay measures the total insulin-stimulated glucose uptake (signal cascade, glucose transport and glucose metabolism) irrespective whether the glucose is utilized via the oxidative or non oxidative pathway. Conversion into lipids, glycogen or membrane-impermeable intermediary products (glucose-6-phosphate) will be detected. Adipocytes are incubated with trypsin (4 mg/mL) for 15 min at 4°C. Soya bean is added to the mixture as trypsin inhibitor. The cells are washed thrice by flotation and used for determination of total uptake glucose. This assay measures the total glucose uptake into cells with inactivated insulin receptor. The first step in the insulin signal transduction cascade (binding of insulin to its receptor) will provide positive results.

Model to study inhibitors of intestinal glucose uptake

The determination of insulin based on the stimulation of glucose uptake by the isolated diaphragm form mice and rats has been used by many investigators to study the effects of insulin and insulin-mimetic substance on muscle tissue.

Intact washed rat diaphragms are incubated (for 30 minutes at 37°C) in HEPES-buffered saline under constant bubbling with 95% O₂ and 15% CO₂. The diaphragms are then washed two times with the same buffer lacking glucose and further incubated (30 min) in 5 mL of glucose free buffer in the presence of test compounds or insulin. Glucose transport is initiated by addition of 10 mM 2(1-3H) deoxyglucose in the absence of presence of 25 DM cytochalasin B (control). After 15 min the diaphragm are rinsed 4 times with icecold buffer containing 10 mM glucose 25 DM.

***In vitro* anti-hyperglycemic activity screening by starch-iodine color assay**

Figure 5 shows a diagrammatic representation of in vitro anti-hyperglycemic activity by starch-iodine colour assay. This assay is based on the slight modification of starch-iodine test. Various concentrations of test solutions are prepared and incubated with alpha amylase containing sodium phosphate buffer (pH around 6.9 containing sodium chloride) at 37°C for 10 minutes. Soluble starch solution (1%) is added to each reaction well and incubated at 37°C for 15 minutes. Hydrochloric acid (1 M) is added to stop the alpha amylase action followed by the addition of iodine reagent. The color change is noted and the absorbance at 660 nM is read in a microplate reader. The same procedure is repeated with control.

From the observation inhibition of enzyme activity is calculated by using the formula:

$$\text{Inhibition of enzyme activity}(\%) = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

Glucose diffusion assay method

This is a simple diffusion method to evaluate the glucose movement *in vitro*. Figure 6 shows a diagrammatic representation of glucose movement by using dialysis membrane. In this method, D-glucose solution is prepared in 0.15 M sodium chloride solution, which is transferred to dialysis tube and mixed with sample to be tested. The other end of tube is closed and the sealed membrane is placed in to the conical flask containing NaCl. The conical flask is placed in the orbital shaker incubator at 37°C and kept at 100 rpm. At various time intervals withdraw the external solution for testing the glucose by using a glucose oxidase kit. From the result, we can plot curve and compare with a standard curve prepared using standard glucose concentrations. Calculate the glucose diffusion retardation index (GDRI) using the following formula:

$$\text{GDRI} = \frac{[100 - \frac{\text{Glucose content in external solution in the presence of test solution}}{\text{glucose content in external solution in the absence of test solution}}]}{100} \times 100$$

Insulin secretion in culture of HIT-T15 cells

HIT-T15 cells are seeded into collagen coated sterile 96 good plate at density of cell per well. After 24 hours' recovery period, the cells are washed with appropriate buffers. Then preincubation is carried out in the same buffers for 30 minutes at 37°C. The buffer is then removed. Prepare different concentration of standard drugs like glibenclamide and then add to the wells. Incubate the cell for 1 h at 37°C. The supernatant is collected and the released insulin is measured using ELISA test.

CONCLUSION

Recently various *in vitro* methods are developed for the screening procedure. *In vitro* methods involve the study of glucose uptake, effect on glycosylation of the hemoglobin and inhibition of various enzymes which are responsible for the carbohydrate metabolism and absorption from the gut such as alpha amylase, alpha glucosidase, sucrase etc. *In vitro* technique like cell culture is one of the important methods to evaluate the activity of newly synthesized and natural compounds. Most of the antidiabetic drugs act on pancreas especially sulfonylurea derivative drug which increases the insulin secretions from beta cells of pancreas. Therefore, the sulfonylurea activity can be evaluated by using the cell lines. High throughput methods for comprehensive evaluation of antidiabetic drugs acting via different mechanisms are warranted at present.

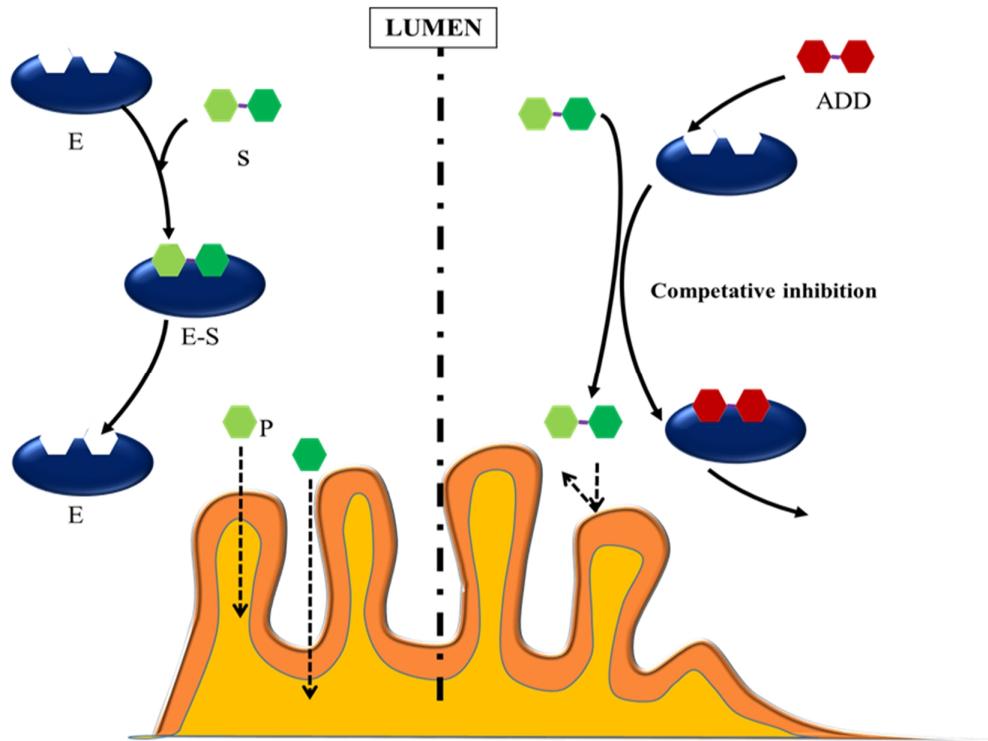


Figure 1: Enzymatic metabolism of carbohydrate in lumen. E:Enzyme (α -amylase, maltase, isomaltase, glucoamylase, sucrase), S:Substrate (starch, maltose, maltotriose, dextrin, sucrose), E-S: Enzyme Substrate complex, ADD: Antidiabetic drug. P: Product (glucose).

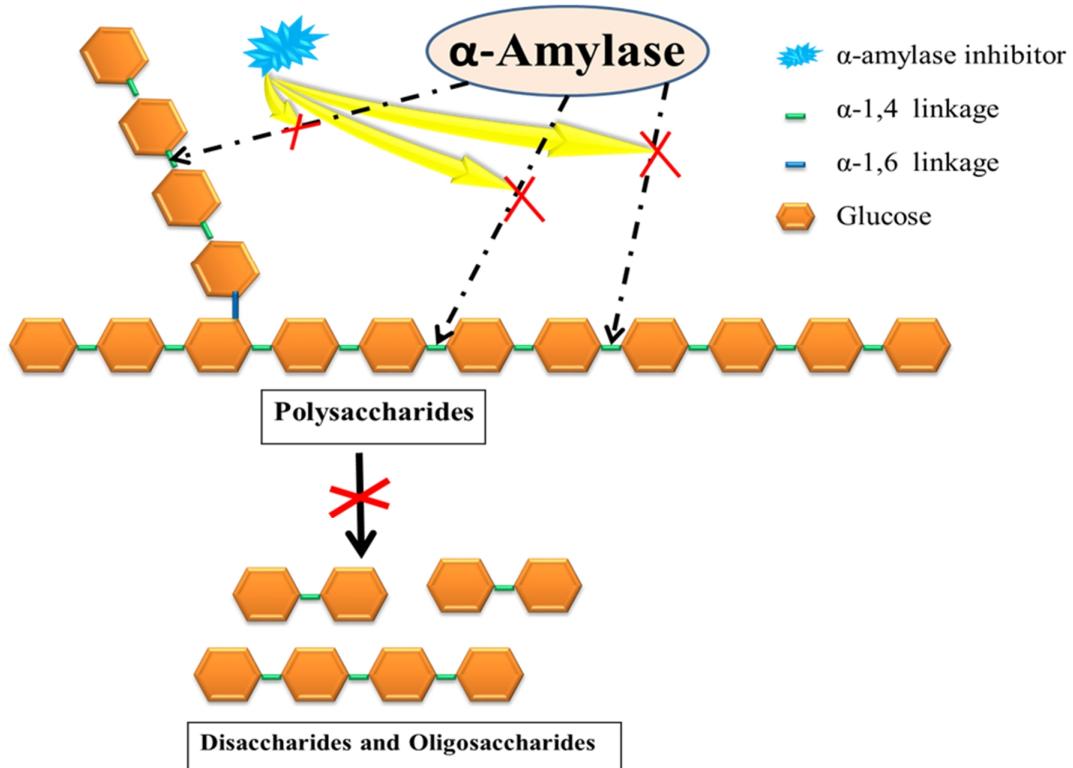


Figure 2: Mechanism of action of α -amylase. The presence of α -amylase inhibitors blocks the enzyme activity and further breakdown to glucose

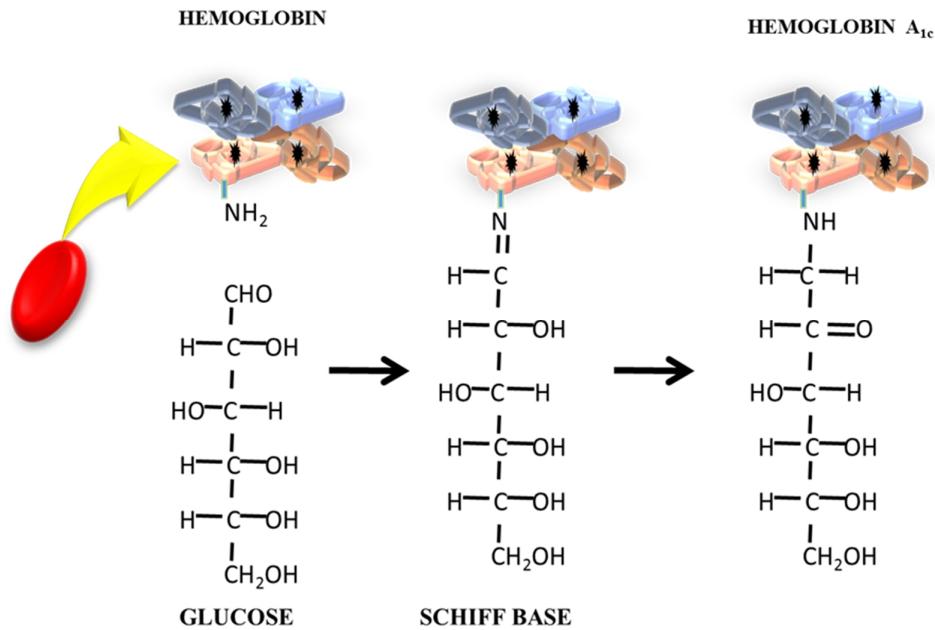


Figure 3: Hemoglobin glycosylation

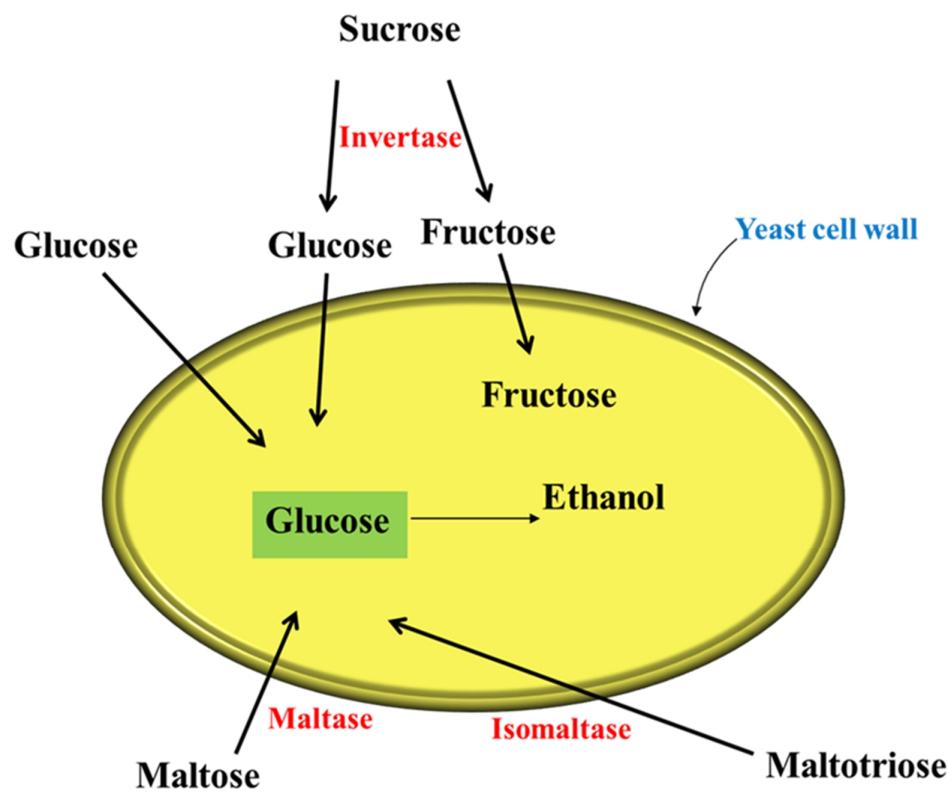


Figure 4: Glucose uptake by yeast cell

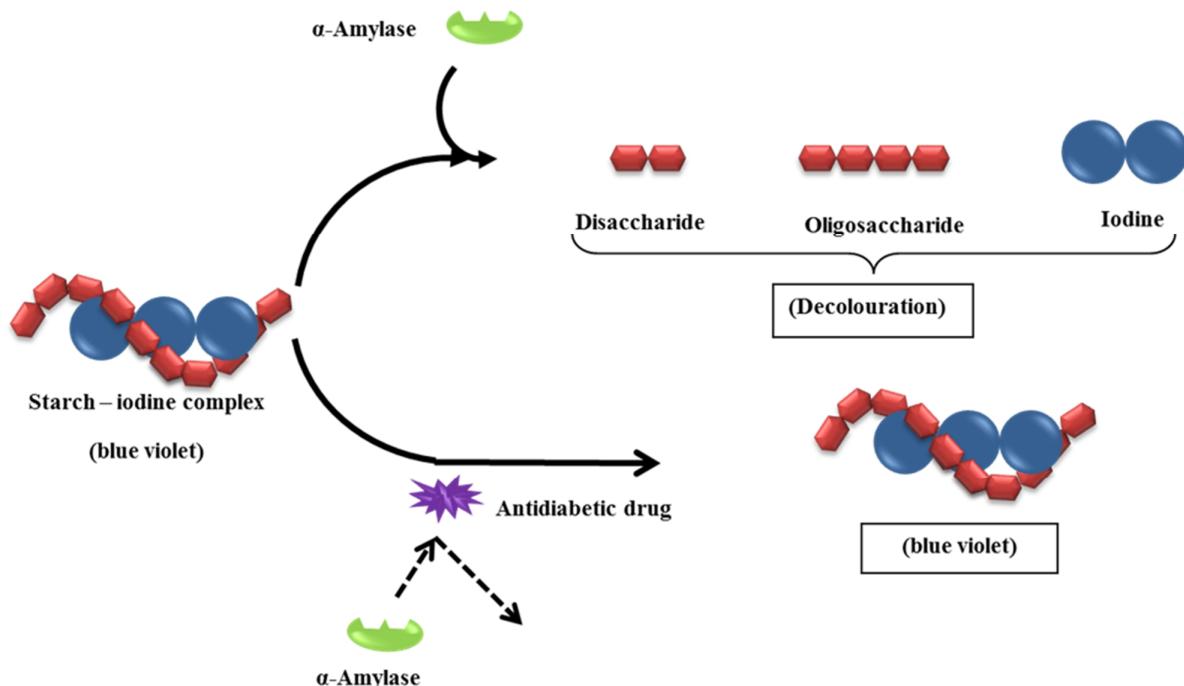


Figure 5: *In vitro* anti-hyperglycemic activity by starch -iodine colour assay

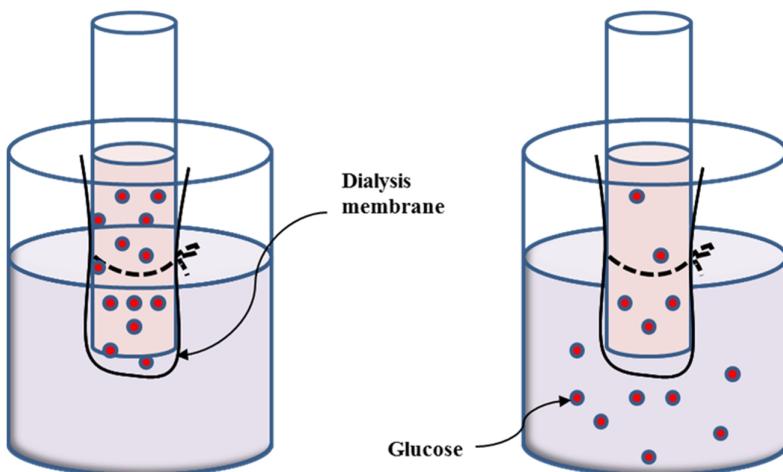


Figure 6: Glucose movement by using dialysis membrane

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