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BEE FREE HONEY PRODUCTION USING *ASPERGILLUS NIGER*

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ABSTRACT

The demand for honey is ever increasing. To meet the demand, adulteration of honey is at its peak. An attempt is made to prepare bee free honey with microbial enzymes. It is aimed to increase the fructose and reduce the glucose from sucrose hydrolysate. *Aspergillus niger* was isolated producing invertase and glucose oxidase. Microbial fermentation conditions are optimized for maximum production of invertase and glucose oxidase. Immobilized *A.niger* and enzymes were used for conversion of sucrose syrup to honey, at 40°C. The prepared honey is characterized by 42.6% fructose and 23.1% glucose and further concentrated to 80 brix with boiling.

Key words:*Aspergillus niger* , 2.Bee free honey 3. Fructose 4. Invertase 5. Glucose oxidase

Introduction:

Honey is defined as the excretions of insects sucking on the living parts of plants (E. Mendes. et al., 1998). Honeybees are the most well-known plant –sucking insects and can collect and transform honey, and deposit, dehydrate, store and leave honey in the honey comb to ripen and mature. Honeybees collect pollen and nectar from a variety of flowering plants and convert it into the wax and honey. Humans have prized honey for its sweet taste for many thousands of years (La Grange & Sanders, 1988). Honey contains vitamins and minerals in trace quantities, including small amount of Iron, Zinc, and Potassium (M.L. González-Miret et al., 2005).Honey composition is influenced by the floral types, climatic and environmental conditions (E. Anklam, 1998; L. Azeredo et al., 2003). Raw honey has been used as a folk remedy throughout history and variety of benefits and medical uses like, it is a food source of antioxidants, it has anti-bacterial and antifungal properties, it can heal wounds, helps for digestive issues, soothe a sore throat and it also have phytonutrient powerhouse(Shamala et al., 2002; Werner and Laccourreye, 2011). But due to deforestation, reduced flowers, number of honeybees, hives are reduced, subsequently natural honey production also reduced. Invertase and glucose oxidase are secreted by the hypo pharyngeal glands of honeybees for the hydrolysis of sucrose and the preservation from microbial effects, respectively. This fact coupled with a higher demand means that honey is becoming an increasingly scarce commodity and consequently, honey adulteration is on the rise. Invertases [β-D-fructofuranosidase (E. C. 3.2.1.26)] catalyze the hydrolysis of a-1,4-glycosidic bonds of sucrose and release equimolar mixtures of monosaccharides D-glucose and D-fructose called invert sugar (Aranda et al., 2006; Mobini-Dehkordi et al., 2008;Veana et al., 2011). Different strains of microbes are used for the production of invertase. The primary strain used for the production of invertase is *Saccharomyces cerevisiae*. Generally, *A.niger, S. cerevisiae, Candida utilis*, are the microbes regarded ideal for invertase production (Klich MA, 2002; Schuster E et al., 2002).Fructo oligosaccharides function as prebiotics and exert advantagious effects on human health (Kurakake et al., 2009; Coman et al., 2012; Rolim, 2015). Glucose oxidase (β -D glucose: oxygen 1- oxidoreductase, EC 1:3:4), an enzyme which oxidizes glucose to gluconic acid. All Aerobic organisms contain this enzyme. Glucose oxidase is generally used as a diagnostic reagent in medicine (Coxon and Schaffer, 1971), and also in estimation of blood glucose level. Aerobic fermentation of *Aspergillus niger* and *Penicilliium* species produces glucose oxidase (Plush Kell et al., 1996; Rando et al., 1997). As both the enzymes, Invertase and glucose oxidase are present in *Aspergillus niger*, we used *A. niger* in the production of invertase and glucose oxidase for bee free honey preparation. *A. niger* synthesize intra-extracellular invertase, which is a significant advantage to food industry, for production of ultra-high fructose syrup from sucrose by a single-step process (Rutherford PP et al., 1972; Camelia NB et al., 2011). In the present study an attempt is made to use *A.niger's* Invertase and Glucose oxidase.

Materials and methods:

Materials: Sugarcane bagasse, Potato dextrose agar, Sucrose and chemicals of lab grade obtained from MERK.

Isolation of fungi for Invertase and Glucose Oxidase production:

Potato dextrose agar was prepared by mixing 500g potato infusion, 20g sucrose and 20g agar in one liter of distilled water. Petri plates and plugged flasks were autoclaved at 15lbs, 110°C for 10 minutes. After cooling, the media was poured into the plates. A small section of sugarcane bagasse (having a black fungal portion) was placed on the potato dextrose agar and incubated at 30°C for 48 hours.

Fermentation:

Enzymes Invertase and Glucose oxidase were produced by submerged fermentation of *A.niger*. The culturing of *Aspergillus niger* was carried out in 250ml flask, containing 50ml of sterile fermentation medium. The composition of fermentation medium (gm/L) was sucrose 50g, yeast extract 10g, peptone 10g, ammonium sulphate 1g, magnesium sulphate 0.45g, potassium dihydrogen phosphate 1.85g,diammonium hydrogen phosphate 0.4g, pH 5.0. 3ml of spore suspension was inoculated into the medium and incubated at 30°C for 5 days in shaker incubator at 100rpm.

Optimization of fermentation conditions:-

Manual optimization of fermentation conditions is carried out by taking and conditioning per time to improve the Invertase and Glucose oxidase production.

Effect of Incubation period:

The fermentation medium was incubated for 120hr at 30°C. For every 24hrs samples were drawn and tested for the activity of glucose oxidase and invertase.

Effect of Incubation temperature:

The fermentation medium was incubated at different temperatures 28°C, 30°C, 32°C, 34°C, 36°C and 38°C. At the end of 5 days, the flasks were checked for glucose oxidase and invertase activity.

Effect of Inoculum volume:

A niger Conidia from 1-week-old potato dextrose agar (PDA) plates were suspended in sterile normal saline solution. A suspension containing 5×10^8 conidia ml^{-1} was used as inoculum. The fermentation medium was inoculated with 1ml, 2ml, 3ml, 4ml, 5ml and 6ml of spore suspension. Glucose oxidase and invertase activity was checked after 5 days.

Effect of pH:

The fermentation medium was subjected to different pH ranges 2, 3, 4, 5, 6, 7, 8 and incubated at 30°c for 5days. After 5 days, the activity of glucose oxidase and invertase was verified.

Effect of shaking:

The fermentation medium was incubated at 30°C for 5 days with varying shaking 50rpm, 100rpm, 150rpm, 200rpm and 250rpm. The activity of glucose oxidase and invertase was verified after 5 days.

Effect of Carbon sources:

Different carbon sources such as glucose, lactose, fructose, sucrose were added to fermentation medium in 1%w/v and incubated at 30°C for 5 days. The activity of glucose oxidase and invertase was verified after 5 days.

Effect of Nitrogen sources:

Different nitrogen sources such as peptone, urea, yeast and malt extracts were added to fermentation medium in

1%w/v and incubated at 30°C for 5days. Thereafter the activity of glucose oxidase and invertase was verified.

Effect of KH_2PO_4:

Different concentrations of KH_2PO_4 0.2%, 0.4%, 0.6%, 0.8% were added to fermentation medium and incubated at 30°C for 5 days. At the end of 5 days, the flasks were checked for glucose oxidase and invertase activity.

Effect of $MgSO_4$:

The fermentation medium was added with different concentrations of $MgSO_4$ 0.01, 0.02, 0.03, 0.04, 0.05 and incubated at 30°C for 5 days. Glucose oxidase and invertase activity was checked after 5 days.

Fermentation under optimized conditions:-

The fermentation was carried out using optimized conditions such as incubation period of 96hrs, incubation temperature of 30°C, inoculum volume of 3ml, at pH 5, at 250rpm, sucrose as carbon source, yeast as nitrogen source, KH_2PO_4 at a concentration of 0.4% and $MgSo_4$ at a concentration of 0.01%.

Enzymes extraction:

After the incubation period (fermentation), the medium was added with tween 80 (1:5 w/v ratio), mixed well on a rotary shaker at 150rpm and filtered. Repeating this process twice, then the filtered suspension was centrifuged at 10000rpm for 10min. The clear supernatant obtained was collected, used as crude enzyme extract for assay studies.

Enzymes assays:

Glucose oxidase: The enzymatic reduction of benzoquinone to hydroquinone in glucose was monitored at 290nm as reported by Cicucu and Patrescu, (1984).

DNS method for invertase: This method involves the oxidation of aldehyde groups of glucose and reduction of 3,5-dinitrosalicylic acid(DNS) to 3 amino, 5-nitrosalicylic acid (at Alkaline pH). The reduced glucose concentrations were monitored at 540nm by UV spectrophotometer as reported by Miller GL, (1959).

Bee free honey preparation:

Bee free honey was prepared by using two methods: 1.The immobilized enzymes, 2. Immobilized *Aspergillus niger*.

Method 1: Invertase and glucose oxidase were immobilized in agar silica cubes. 0.7% agar agar, 0.6% silica gel and 1% gluteraldehyde in water were boiled at 90°C for 10minutes, and then cooled to 50°C. 10% crude enzyme was added, mixed and poured into plate. After solidification it was cut into small cubes. Bee free microbial honey preparation was done by taking 1L of 20% sucrose syrup, adding 150g enzyme cubes prepared above, incubated at 40°C for 1hr. Removed beads by filtration and concentrated by boiling to get 80 brix.

Method 2: *A. niger* was immobilized by taking 2.5% sodium alginate in water and mixed with 2% *A. niger* microbial suspension, 0.5% glycerol and dropped in ice cold 2M cold $CaCl_2$. Bee free microbial honey preparation was prepared by taking 1L of 20% sucrose syrup, adding 150g *A. niger* beads, incubating at 40°C for 1hr.

Characterization of bee free Honey:

Density test:

a) Blot test- Honey was allowed to flow on blot paper. If honey flows on the blot paper without wetting it, then it is regarded ,As pure whereas if honey gets wets the

blotting paper, then it is regarded as adulterated honey.

b) Vinegar and water test- A spoon of Honey is mixed with two spoons of vinegar and small amount of water, if foams appear it is adulterated honey, if foam doesn't appear it is pure honey.

c) Heat test – Honey is heated in this process. when honey is heated, if it quickly broils then it is considered as pure honey, if bubbles appear then it is considered as adulterated honey.

d) Water drop test- In a glass of water, a spoon of honey is dipped; if the honey is dissolving in water then it's adulterated. If the honey has a thick texture which settles at the bottom of a glass, then it is pure honey.

Seliwanoff's test:

In a test tube, 1ml of honey was taken; next 2ml of Seliwanoff's reagent (Dissolve 50 mg resorcinol in 33 ml concentrated HCl and make it to 100 ml with water) was added to it and heated on the flame.

Acidity:

The total acidity was determined by the titrimetric method as follows: The addition of 0.05 M NaOH was stopped at pH 8.50 (free acidity), immediately a volume of 10 mL 0.05 M NaOH was added, without delay, back-titrated with 0.05 HCl to pH 8.30 (lactonic acidity). Results were expressed in meq NaOH kg.

High pressure liquid chromatography (HPLC) of honey samples:

Sugars, gluconic acid and HMF were estimated using HPLC chromatography. The system comprised waters HPLC with a model 600E solvents delivery system, 410 differential refractometer detectors and Millenium 2010 software. An Aminex HPX-87P 3.9X300mm column at 85°C was used. The mobile phase contained deionized filtered water (0.45 mm, nylon membranes, Millipore, Ma, USA) at a flow rate of 0.6ml/min. All standard sugars (sucrose, glucose and fructose), gluconic acid and HMF were obtained. All honey samples were verified for carbohydrates, gluconic acid and HMF in triplicates.

Results:

Isolation of Invertase and glucose Oxidase producing *Aspergillus niger*:

A. niger producing invertase and glucose oxidase was isolated from sugarcane bagasse on potato dextrose agar. Five *A. niger* strains were isolated with following colony and microscopic characteristics.

Colony characteristics: *A. niger* was grown on Potato dextrose agar, displayed a colony of diameter 65-72mm, cottony appearance, white colour during earlier days and later acquired black colour due to spore production.

Microscopic characteristics: It had conidiophores and conidia which were black colored. The conidial heads looks radial and split into columns.

Fermentation:

The enzymes invertase and glucose oxidase were produced by submerged fermentation of *A. niger*. Here, under non-optimized conditions the enzymes were produced. Under non-optimized conditions, the production of invertase and glucose oxidase was 35U/ml, 22U/ml respectively.

Optimization of fermentation conditions:-

Effect of Incubation period:

It is observed that as the incubation period increases, the activity of glucose oxidase and invertase increased till 96hrs, from

there, the activity decreased. The reason behind this was the less availability of nutrients and piling up of toxic materials.

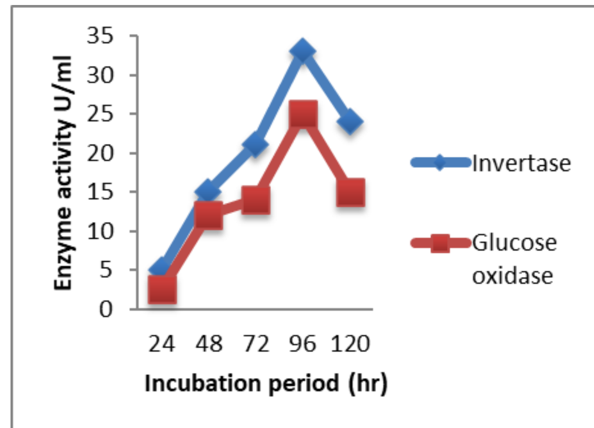

Fig.1: Effect of incubation period on enzymes production

Effect of Incubation temperature:

In this study, it is observed that maximum glucose oxidase and invertase production was at 30oC. At other temperatures, there was varying enzymes production as shown in fig.

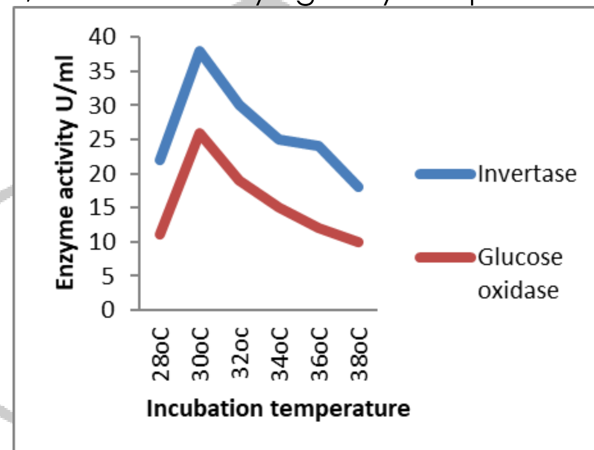

Fig.2: Effect of incubation temperature on enzymes production

Effect of Inoculum volume:

As the volume of inoculum increases, the enzyme activity increases till 3ml, from there it decreased and remained constant.

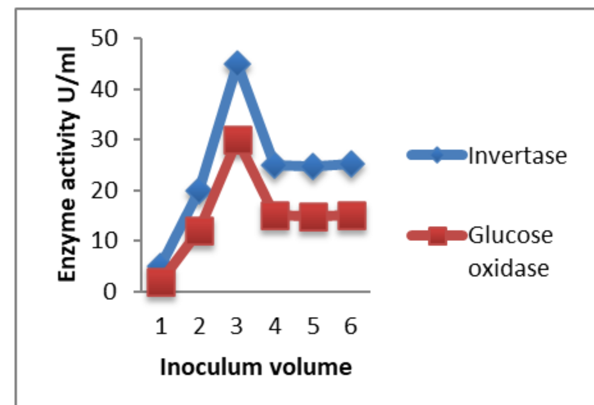

Fig 3: Effect of inoculum volume on enzymes production

Effect of pH:

The maximum activity of the enzymes was at pH 5 and 6.

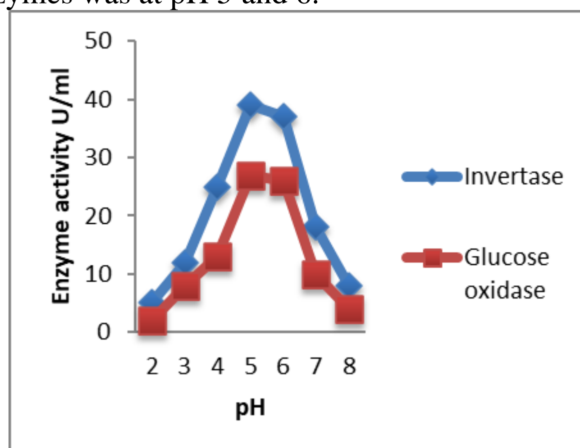

Fig 4: Effect of pH on enzymes production

Effect of shaking:

From 50rpm to 250rpm, the enzyme activity increased in an ascending order and the maximum enzyme activity was at 250rpm. Hence, agitation has an impact on enzyme production.

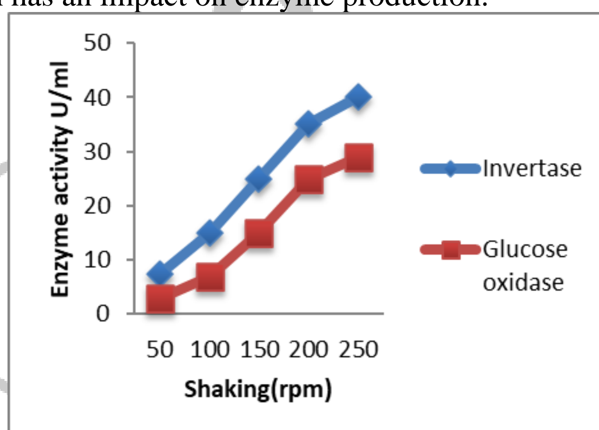

Fig 5: Effect of agitation on enzymes production

Effect of Carbon sources:

We observed that sucrose results in an increased production of invertase and glucose results in the increased production of glucose oxidase.

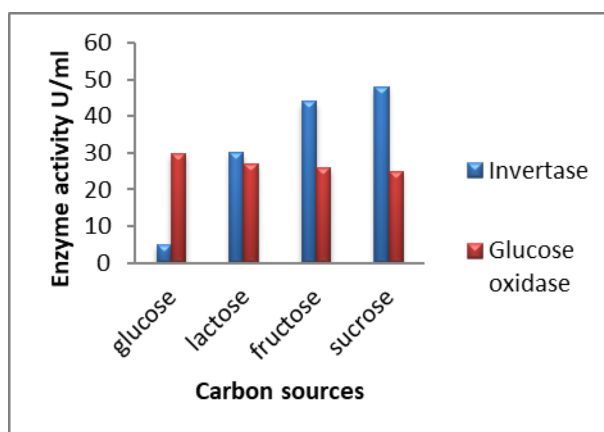

Fig 6: Effect of carbon sources on enzymes production

Effect of Nitrogen sources:

In this study, yeast extract as a nitrogen source has the highest impact on enzymes production.

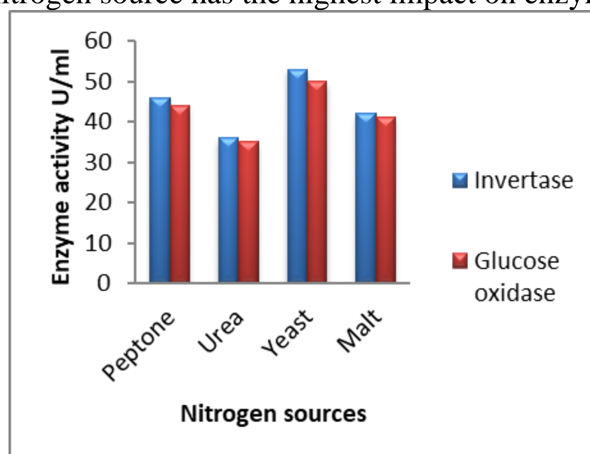

Fig 7: Effect of nitrogen sources on enzymes production

Effect of potassium dihydrogen phosphate (KH_2PO_4):

The maximum enzymes activity was at 0.4% concentration and decreased thereafter as displayed in the figure (8).

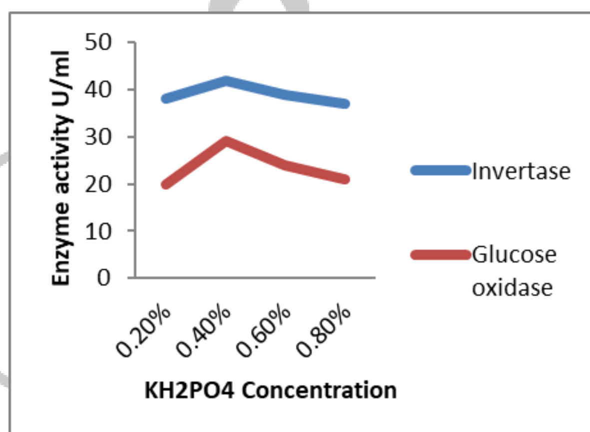

Fig 8: Effect of potassium dihydrogen phosphate on enzymes production

Effect of magnesium sulfate $MgSO_4$:

As the concentration of magnesium sulphate increases, the enzymes production decreases. So, a lowest concentration of 0.01 was found preferable.

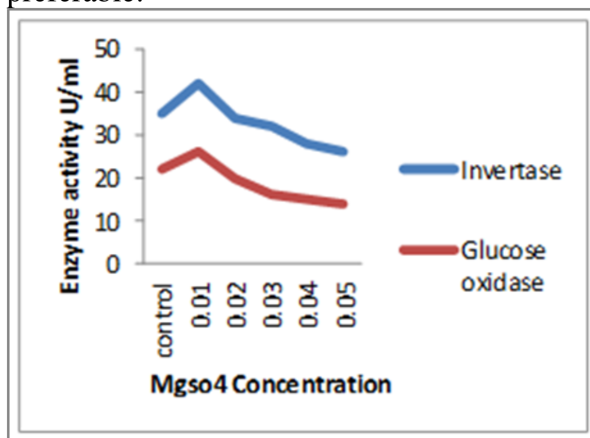

Fig 9: Effect of magnesium sulfate on enzymes production

Fermentation under optimized conditions:

The highest production of enzymes was at incubation time of 96hrs, incubation temperature of 30°C, inoculum volume of 3ml, pH 5-6, agitation of 250rpm, and sucrose as carbon source for invertase, glucose as carbon source for glucose oxidase, yeast extract as nitrogen source, 0.4% of potassium dihydrogen phosphate and 0.01% of magnesium sulphate.

As the fermentation was carried out in optimized conditions, the production of invertase and glucose oxidase was 65, 49, whereas under non-optimized conditions the production of invertase and glucose oxidase was 35, 22. It indicates that under optimized conditions, the production of enzymes was high.

Bee free honey preparation:

Using two methods, honey was prepared. The appearance, texture, colour, taste and nutritional value of the honey also resembled the natural one.

Characterization:

Density tests:

1. Blot test: In the blot test, bee free honey went on the paper without wetting it as wild honey, which indicates its purity.
2. Vinegar and water test: When vinegar and water was added to honey, foam didn't appear as wild honey, which represents the pure nature of bee free honey.
3. Heat test: When the bee free honey was heated, it quickly caramelized as wild honey, hence the honey is pure.
4. Water drop test: The bee free honey had a thick texture which settled at the bottom of a glass as wild honey, displayed its purity.

Seliwanoff's test:

This test is used to verify the presence of ketose in honey. On addition of seliwanoff's reagent and heating, the mixture turned into red colour which indicates the presence of ketose and indicates the presence of fructose.

Acidity:

The values of total acidity present in different honey samples was 29meq/kg (pure honey/control), 28 meq/kg (bee free honey). The sample was in the safe range, which represents the non-appearance of undesirable fermentation.

High pressure liquid chromatography (HPLC) of honey:

Sugars and gluconic acid composition analyses of honey samples was carried out by HPLC. The samples (natural honey, bee free honey) had 5.21% and 4.52% of sucrose respectively. 35.21%, 23.1% was the content of glucose in natural honey and bee free honey respectively. The fructose content was 40.05%, 48.6% in natural honey and bee free honey respectively. Gluconic acid content was 1.2 %in natural honey whereas 11.2% in bee free honey. HMF content was 4mg/kg in natural honey and 55mg/kg in bee free honey. Honey mostly contains the monosaccharide's glucose and fructose. The ratio of fructose to glucose depends largely on source of nectar. Both samples contained high fructose than glucose. Honeys with high fructose/glucose ratios remain liquid for longer periods. The fructose to glucose ratio affects the honey flavor, as fructose is sweeter than glucose. The carbohydrate composition analyses of honey samples were given in table 1.

Table 1: HPLC analysis of honey samples

Sample	Fructose (%)	Glucose (%)	Sucrose (%)	Fructose/Glucose	Gluconic acid (%)	HMF (mg/kg)
Natural honey	40.05	35.21	5.21	1.13	1.2	4
Bee free honey	42.6	23.1	4.52	1.84	11.3	55

Discussion:

Honey is the natural sweet product of *Apis melifera* bees obtained from plant nectars. Deforestation is in the momentum to meet the requirements of increased population and subsequently bee populations are depleted. Decreased bees honey production and increased usage has yielded huge demand. To meet the demand honey is being adulterated with sugary syrups. In the present study microbial enzymes invertase, glucose oxidase was used for honey preparation. *A.niger* was used for glucose oxidase (Liu et al., 1998) and invertase production (Klich MA, 2002) by fermentation. In present study, *A.niger* isolated and used for production of both invertase and glucose oxidase. Invertase converts sucrose to glucose and fructose (Mojdeh Dinarvand et al., 2017), whereas, glucose oxidase converts glucose to gluconic acid. Gluconic acid has been designed as GRAS by USFDA and it is a part of honey (Lucina et al., 2018). Gluconic acid imparts refreshing sour taste (Poonam Yadav et al., 2022). In present study, the carbohydrate composition analysis of honey samples was fructose (42.6%), glucose (23.1%) and sucrose (4.52%). Carbohydrate mean composition in samples of earlier verified honeys in dry and rainy seasons was fructose (38.5% - 44.5%), glucose (29.2%-38.7%), sucrose (2.21%-5.52%) and arabinose (0.03%-0.9%)(Graciela Ojeda de Rodrı´guez et al., 2002). Mean sugars content of different honey samples of the Asir province of Saudi Arabia was fructose (33.97), glucose(29.51), sucrose(3.25) and maltose(2.97)(Hamed A. Ghramh et al., 2020). Amount of sugars in honey determined by HPLC was fructose (25.2), glucose (25.5), and sucrose (3.7) (Jung-Hyeon Yoon et al., 1997). Maximum production of enzymes was achieved at incubation time of 96hrs, incubation temperature of 30°C, inoculum size of 3ml, pH 5-6, agitation of 250rpm, sucrose as carbon source, yeast extract as nitrogen source, 0.4% of potassium dihydrogen phosphate and 0.01% of magnesium sulphate. Maximum production of glucose oxidase was obtained at 10% glucose concentration, incubation time of 48hrs, glucose as carbon source, pH of 5.5, 0.2% of urea and 0.4% of potassium dihydrogen phosphate (Shazia Khurshid et al., 2009). Highest production of invertase was achieved at an incubation time of 96hrs incubation temperature of 30°C, inoculum volume of 3ml, pH 5, and sucrose as carbon source and yeast as nitrogen source (Asha-I Raju CH et al., 2016). The maximum synthesis of invertase was

obtained at incubation period of 120hr, pH 5, incubation temperature of 35°C, sucrose as carbon source and peptone as nitrogen source (Olaoluwa Oyedeji et al., 2017). Production of invertase increased at optimized conditions (pH 6.5, temperature 30 ◦C, 6% (v/v), inoculum size and 150 rpm agitation speed)(Mojdeh Dinarvand et al.,2017). *A.flavus* was produced at high levels at 96hrs of incubation time, pH 5, incubation temperature of 30oC, inoculum volume of 3 %(C. Uma. et al., 2010). In this study, prepared bee free honey using the enzymes invertase and glucose oxidase produced by *A.niger*. A team from Department of Biotechnology and Food Engineering at Israel's the Technion - Israel Institute of Technology has developed recombinant *Bacillus subtilis* which produces bee free honey (Crop biotech update, 2019). In this study, bee free honey was proved pure in all density tests, showed the presence of ketose, HMF (55mg/kg), acidity (28 meq/kg). Honey characterized by Rosane da S. Sant'ana et al., 2020 showed moisture content (27.2), acidity (23-28 meq/kg), HMF (2 (mg Kg−1 - 4.4mg Kg−1). Honey characterized by Luís R. Silva et al., 2009 showed moisture content of 13.5 – 19.7; acidity was below 50meq/kg, HMF of 1.75 to 32.75 mg/kg and six samples showed diastase activity. From the analysis of bee free honey it was found similar to wild honey with reduced glucose content and little increase in gluconic acid and HMF content. Gluconic acid is also a beneficial compound whereas HMF has increased very less.

Conclusion

An attempt is made to prepare bee free honey with microbial enzymes. *Aspergillus niger* was isolated producing invertase and glucose oxidase. Microbial fermentation conditions are optimized for maximum production of invertase and glucose oxidase. Immobilized *A.niger* and enzymes were used for conversion of sucrose syrup to honey, at 40°C. The prepared honey is characterized by 42.6% fructose and 23.1% glucose and 80 brix.

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