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Chemically Defined Medium for the Production of Phytase by *Hanseniaspora guilliermondii* S1, *Pichia fermentans* S2 and its Secondary Structure Prediction of 16S rRNA

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Abstract: Chemical efficiency and medium formulation was the essential step for design highly demands laboratory experiments for the yield enhancement and productivity of phytase by two strains of *Hanseniaspora guilliermondii* S1 MG663578 and *Pichia fermentans* S2 MG663579.The SSF experimental model showed maximum phytase production of 365 U/ml appearing g/100ml: Phytic acid (1.5), peptone (0.15), dextrose (0.50), yeast extract (0.05), malt extract (0.05) pH 5.5 and 28^0C used 10^8 cells ml-1 culture *Hanseniaspora guilliermondii* S1 MG663578.In enzyme kinetics, Km and Vmax values were found to be 3.3 mM and 0.4 µmol/min using phytic acid as a substrate in *Hanseniaspora guilliermondii* S1 MG663578.The secondary RNA structure of active strain *Hanseniaspora guilliermondii* S1 MG663578 was predicted 15 stems in their MFE and Centroid secondary structure and dot-bracket notation showed that the free energy of the structure was - 165.7 kcal/mol; the threshold energy is -4.0 with cluster factor, conserved factor 2 and compensated factor 4; and the conservatively is 0.8.

Keywords: *Hanseniaspora guilliermondii* S1; RNA; Entropy; thermodynamics free energy ; Solid state fermentation; MFE secondary structure.

1. Introduction

Phytases (EC 3.1.3.8) are commercially named Natuphos, which was primary introduced into the global market in 1991 [1]. Phytases are special classes of phosphomonoesterases, catalyze the hydrolysis reaction of phytate to the Mono-, Di-, Tri-, Tetra-, and Pentaphosphates of *myo*-inositol and inorganic phosphate. Hydrolysis with 3-Phytases (yeast) is initiated at C3 carbon of the ring yielding (1, 2, 4, 5, 6) IP5 [2]. Hence, microorganisms are the preferred sources of phytases due to high yield, easier production and the ease of handling and manipulation of producing strains. In view of yeast are the most dominant phytase producers as compared to several strains of fungi and yeast [3-4].

The phytase production by yeast has been successfully bringing about using solid state fermentation [5]. Though there are various sources of phytase, microbes are the organisms of choice for producing the enzyme for various applications [6].Several investigators have optimized the and physical parameters for maximizing the production of yeast phytases [7-9]. Solid State Fermentation (SSF) has higher important advantages than submerged fermentation

(SmF) because of simpler defined fermentation medium,low water content and minimize contamination from microbes [10].

The structures of RNA molecules are often important for their function and regulation in the growth of yeast. The finding that RNA secondary structure from thermodynamics could possess functionality once believed to be the sole domain of protein enzymes, led to the hypothesis that RNA could have preceded protein and DNA in an RNA.RNA may have evolved and may continue to evolve many yet unknown functions. Evolution may be fundamental chemical principles slow to eliminate non-functional RNAs. RNAs without current function, however, may constitute a pool of molecules that might be adapted to fill novel roles [11].

It is well known that production of phytase from yeast can be influenced by a number of nutritional factors, such as the amount of peptone, dextrose in the medium as carbon sources, yeast extract as nitrogen sources, demonstrated that addition of 0.5 % phytic acid to the culture medium enhanced phytase production by yeast in a solid-state fermentation. They also observed that substrate concentration in the medium had an effect on phytase production. In this work we carried, optimal secondary structure, free energy of the thermodynamic ensemble of *Hanseniaspora guilliermondii* S1 (MG663578) and *Pichia fermentans* S2 (MG663579) were analysed. Optimal chemical constituents of the medium were assed for most effective nutrients to enhance phytase production based on the enzyme activity activity and growth.

2. Materials and Methods

2.1. Media and chemicals.

Peptone, dextrose, yeast extract, malt extract, sodium nitrate, magnesium sulphate, ferrous sulphate, BSA and agar from Hi Media, India.Phytic acid sodium salt and Manganese sulphate from TCI, India.

2.2. Maintenance of culture.

Identified yeast cultures were stored in PG and Research centre in Biotechnology, MGR College, Hosur, Tamilnadu, India. Pure cultures of two strains were obtained under standard operating protocols. The following *Hanseniaspora guilliermondii* S1 (MG663578) and *Pichia fermentans* S2 (MG663579) strains have been used for further experiments.

2.3. Secondary structure prediction of 16S rRNA.

The secondary structures of *Hanseniaspora guilliermondii* S1 (MG663578),*Pichia fermentans* S2 (MG663579) was predicted using the bioinformatics tools available in online RNAWebSuite/RNAfold. and energy dot plot analysis by http://rna.tbi.univie.ac.at. The following links were used [12]: S1 secondary structure (link); S2 secondary structure (link); S1 and S2 RNA-RNA interaction (link);

2.4. Restriction site analysis.

The restriction sites in 16S rRNA of *Hanseniaspora guilliermondii* S1 (MG663578), *Pichia fermentans* S2 (MG663579) was analysed using NEB cutter programme version 2.0. The following links were used [13]: S1 NEB single cutter restriction enzymes (link); S2 NEB single cutter restriction enzymes (link);

2.5. Sub culturing.

The identified strains S1 - *Hanseniaspora guilliermondii* (MG663578), S2- *Pichia fermentans* (MG663579) were subculture and grown initially on YEPMD broth. It contains (g/100ml): Dextrose (2 g), yeast extract (1 g), malt extract (1 g) and peptone (1g) kept in refrigerator until use.

2.6. Formulation of Chemical defined medium.

2.6.1. Effect of different concentrations of peptone on Phytase Production.

To detect the appropriate carbon source for phytase production by the isolates, the fermentation medium was supplemented with five concentration of peptone (0.15-0.55g/100mL) and add 1 ml (10^8 cells mL−1) *Hanseniaspora guilliermondii* (MG663578), S2-*Pichia fermentans* (MG663579) separately and incubation was carried out on 150 rpm rotary shaker at 28°C for 72h.

2.6.2. Effect of different concentrations of dextrose on Phytase Production.

To detect the appropriate carbon source for phytase production by the isolates, the fermentation medium was supplemented with five concentration of dextrose (0.25-1.25g/100ml) and add 1 ml (10^8cells mL−1) *Hanseniaspora guilliermondii* (MG663578), S2-*Pichia fermentans* (MG663579) separately and incubation was carried out on 150 rpm rotary shaker at 28°C for 72h.

2.6.3. Effect of different concentrations of Yeast extract on Phytase Production.

To detect the appropriate nitrogen source for phytase production by the isolates, the fermentation medium was supplemented with five concentration of yeast extract (0.05-0.25g/100ml) and add 1 ml (10^8cells mL−1) *Hanseniaspora guilliermondii* (MG663578), S2-*Pichia fermentans* (MG663579) separately and incubation was carried out on 150 rpm rotary shaker at 28°C for 72h.

2.6.4. Effect of different concentrations of Malt extract on Phytase Production.

To detect the appropriate nitrogen source for phytase production by the isolates, the fermentation medium was supplemented with five concentration of malt extract (0.05-0.25g/100ml) and add 1 ml (10^8cells mL−1) *Hanseniaspora guilliermondii* (MG663578), S2-*Pichia fermentans* (MG663579) separately and incubation was carried out on 150 rpm rotary shaker at 28°C for 72h.

2.6.5. Effect of different concentrations of substrate on Phytase Production.

To detect the appropriate substrate source for phytase production by the isolates, the fermentation medium was supplemented with five concentration of phytic acid (0.25-1.25g/100ml) and add 1 ml (10^8cells mL−1) *Hanseniaspora guilliermondii* (MG663578), S2-*Pichia fermentans* (MG663579) separately and incubation was carried out on 150 rpm rotary shaker at 28°C for 72h.

2.6.6. Effect of different concentrations of substrate on Phytase Production.

The effect of temperature on phytase production was investigated at 28,30,32,37 and 40°C for 72 h. The effect of initial pH on phytase production was investigated by pH to 5.5,6.5,7.5,8.5 and add 1 ml (10^8cells mL−1) *Hanseniaspora guilliermondii* (MG663578), S2-*Pichia fermentans* (MG663579) separately and 9.5 on 150 rpm rotary shaker at 28°C for 72 h.

2.7. Optimized chemical constituents.

After optimizing the production of phytase under shake flask conditions. The sources of peptone 0.15%, dextrose 0.5%, yeast extract 0.05%, malt extract 0.05% ,phytic acid 0.5%, 0.05% $NaNO_3$, 0.05% $MgSO_4.7H_2O$, 0.05% KCl, 0.001% $FeSO_4.7H_2O$, 0.001% $MnSO_4.6H_2O$ and add 1 ml of 10^8cells mL−1) culture, condition was maintained at pH 5.5 and temperature 28°C separately.The shaker was run with agitation 150 rpm for 72h.Samples were withdrawn at regular intervals and analyzed for protein content and phytase assay. After incubation, the production medium was centrifuged at 15,000 rpm for 15 min at 4^0C and the supernatant was collected and used for phytase activity assay by (trichloroacetic acid, Taussky shorr color reagent) [14].

2.8. Enzyme kinetics by nonlinear fitting with system function.

Enzyme kinetic parameters of phytase were obtained by measuring the rate of phytate hydrolysis at various substrate phytic acid concentrations ranging from 0.1 to 3.3 mM in the standard reaction mixture (50 mM sodium acetate buffer (pH 4.0) at 85°C). The Michaelis–Menten constant (Km) and maximum velocity (Vmax) values were determined from nonlinear fitting with system function by Lineweaver–Burk plot using software (Origin 8.0 SR6).

3. Results and Discussion

3.1. Secondary RNA structure.

The secondary RNA structure of active strain *Hanseniaspora guilliermondii* S1 MG663578 has predicted 15 stems in their MFE and Centroid secondary structure (**Figure.1-2**). This prediction dot-bracket notation showed that the free energy of the structure is - 165.7 kcal/mol; the threshold energy is -4.0 with cluster factor, conserved factor 2 and compensated factor 4; and the conservatively is 0.8.Similar results attained by the author [15].

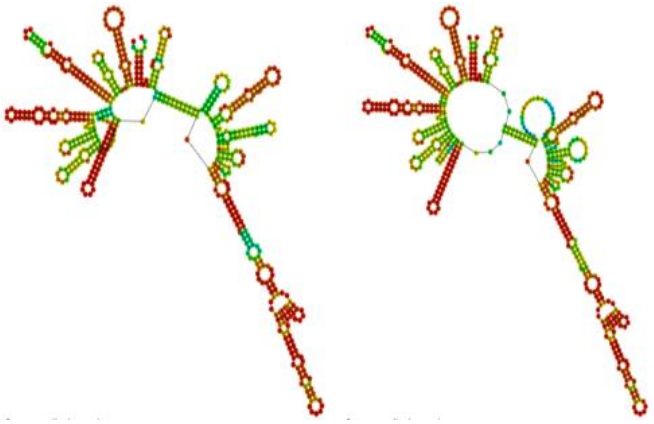

Figure 1. MFE secondary structure, Centroid secondary structure of MG663578.

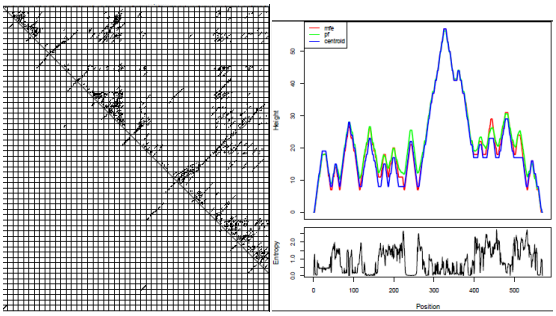

Figure 2. Dot and entropy mountain plot of MG663578.

The secondary RNA structure of active strain *Pichia fermentans* S2 MG663579 has predicted 13 stems in their MFE and Centroid secondary structure (**Figure 3-4**). This prediction dot-bracket notation showed that the free energy of the structure is -175.1 kcal/mol; the threshold energy is -3.0 with cluster factor, conserved factor 3 and compensated factor 6; and the conservatively is 0.18.

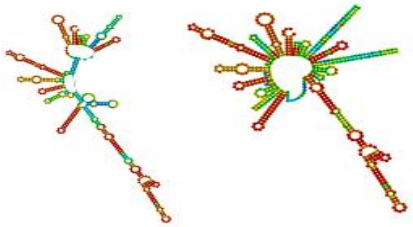

Figure 3. MFE secondary structure, Centroid secondary structure of MG663579.

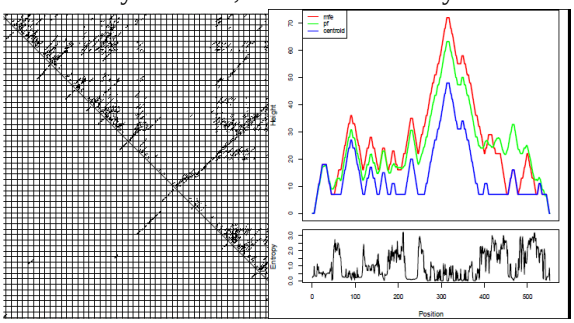

Figure 4. Dot and entropy mountain plot of MG663579.

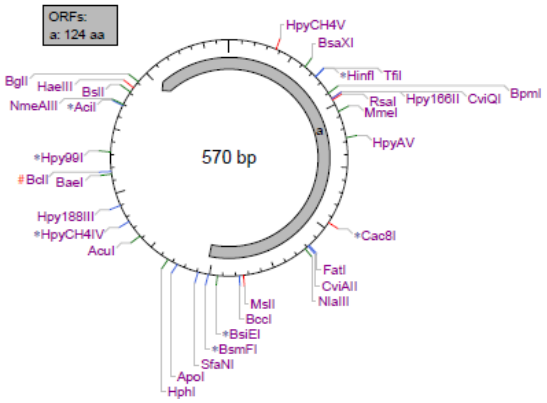

Figure 5. Circular Sequence of MG663578.

3.2. Prediction of restriction sites.

The prediction of restriction sites found in the strain *Hanseniaspora guilliermondii* S1 MG663578 shown in Figure 5. Totally, 32 restriction enzyme sites were observed, such as HpyCH4V,BsaXI,Tfil,Hinfl,Hpy166II,cviQl,Rsal,Mmel,HpyAV,caabI,Fatl,CviAII,Nlall,Msll

,Bccl,BsiEl,SfaNl,Apol,Hphl,Acul,HpyCH4IV,Hpy188lll,Bael,Bcll,Hpy99l,Acil,NmeAlll,Bsl l,Haelll and Bgll. The GC,AT contents of *Hanseniaspora guilliermondii* S1 MG663578 was found to be 42% GC and 58%ATrespectively.

The prediction of restriction sites found in the strain *Pichia fermentans* S2 MG663579 shown in Figure 6. Totally, 35 restriction enzyme sites were observed, such as HpyCH4V,EcoO1091,Faul,Ecop151,Accl,Hpy166ll,Bbvl,CViQl,Rsal,Tsel,ApeKI,Eael,Eagl, HpyAV,Stul,Hgal,Avall,Msll,Bcc,BsmFl,Apol,Hphl,BBsl,Acul,Styl,HpyCh4IV,Hpy188lll,Ta ql,Alwl,Bael,Bcll,Hpy991,NmeAlll,BssHll and BstUl. The GC,AT contents of *Pichia fermentans* S2 MG663579 was found to be 47% GC and 53%AT respectively. Similar results attained by the author [16].

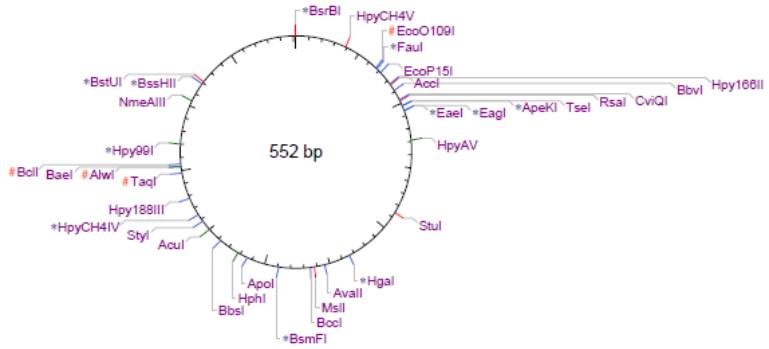

Figure 6. Circular Sequence of MG663579.

The medium formulation is the essential step for a design highly demands laboratory experiments for the yield enhancement and productivity.The nutritional constituents of the medium must satisfy the elemental requirement for cell biomass and enzyme production, however,it can be adequate energy supply for biosynthesis and cell maintenance of industrial. During the fermentation, optimization in this context needs careful consideration of the physical and nutritional parameters. Growth and production process for fermentation that is used to grow cells, it is necessary to monitor and control parameters for starting from the optimum carbon and nitrogen sources and including pH, temperature and incubation period. Changes in one of these parameters can have an ideal effect on the yield of enzyme and the stability of growth. The high rate of metabolism supports the critical period of enzyme production [17].

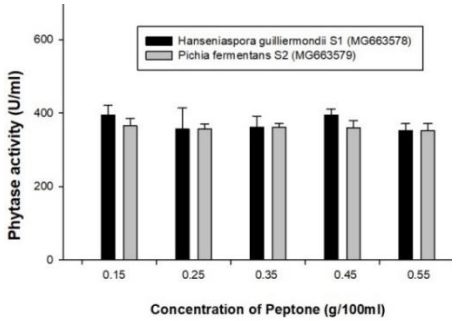

Figure 7. Different concentration of peptone.

3.3. Experimental design for production.

3.3.1. Effect of peptone on phytase production.

The effect of supplementation of the source with 0.15-0.55 g/100ml of different concentrations of peptone was added. It was found that 0.15 concentration achieved the most

effective phytase productivity for the strain *Hanseniaspora guilliermondii* S1 MG663578 (395±4.1) where as *Pichia fermentans* S2 MG663579 (365±4.6) **(Figure 7)**. These results agree well with existing studies on peptone consumed by microorganisms. The observations also agree with the results peptone supported phytase production reported [18].

3.3.2. Effect of dextrose on phytase production.

The solid medium was supplemented with dextrose as carbon source concentration of 0.25 to 1.25g/100ml.The results revealed that 0.50g/100ml concentrated was produced highest activity of phytase in *Hanseniaspora guilliermondii* S1 MG663578 (395±8.2) whereas *Pichia fermentans* S2 MG663579 (365±2.8) **(Figure 8)**. The results are consistent with phytase production reported [19].

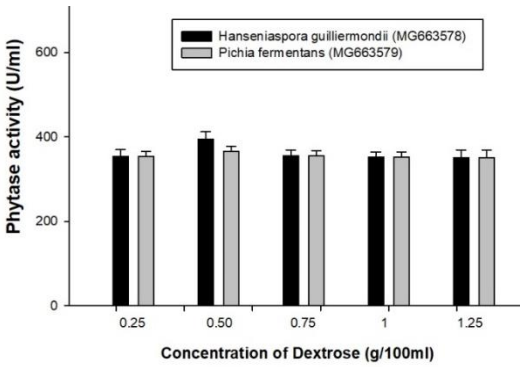

Figure 8. Different concentration of dextrose.

3.3.3. Effect of yeast extract and malt extract on phytase production.

The production medium supplemented with (0.05-0.25g/100ml) of two different nitrogen sources included. The results show that the addition of 0.05g/100ml yeast extract and malt extract separately resulted in great enhancement of phytase production by *Hanseniaspora guilliermondii* S1 MG663578 (395±6.7) whereas *Pichia fermentans* S2 MG663579 (365±3.2) **(Figure 9-10)**. Most of the yeast can utilize inorganic or organic nitrogen sources. Inorganic nitrogen may be supplied as ammonium salts ammonia gas, or nitrates and as protein or urea. It was found that the growth was faster with the supply of organic nitrogen, and a few microorganisms also were found to have an absolute requirement for nitrates. The study of different organic and inorganic nitrogen sources appeared that yeast extract was the most favorable nitrogen source for enzyme production. On the other hand, reported that malt extract was the most favorable nitrogen source for *Pichia anomola* phytase production [20].

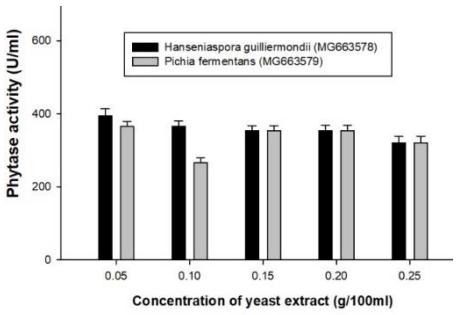

Figure 9. Different concentration of yeast extract.

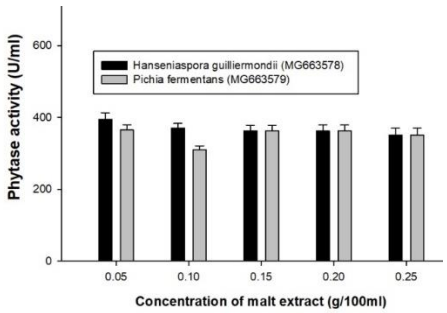

Figure 10. Different concentration of malt extract.

3.3.4. Effect of substrate on phytase production.

It was found that such an approach can produce phytase by using chemical substrate in Solid state fermentation. The effect of supplementation of the substrate with 0.5-2.5 g/100ml of different concentrations of phytic acid was added. The results in **Figure 11** showed that the best concentration was reached at substrate 1.5 g/100ml and phytase activity was found *Hanseniaspora guilliermondii* S1 MG663578 (395±2.7) where as *Pichia fermentans* S2 MG663579 (365±1.1).Yeast is considered the better adapted organisms for solid state fermentation, since their spore could penetrate on the surface of particles and are also able to grow easily and then colonized the entire medium. Using of chemical substrate in SSF became the main theme of several researchers, to get low cost medium and solve the pollution problems resulted from the accumulation of these residues. Many studies consistently report the isolate was cultivated on different as substrates. The results in the present work states that phytase production with this suitable substrate [21]. The chemical substrate that provides all the required nutrients to the yeast growing in it should be considered as the ideal substrate in this present study.

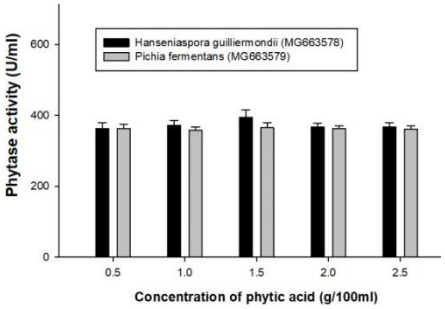

Figure 11. Different concentration of phytic acid.

3.3.5. Efficacy of temperature and pH.

Two key factors needed to be accounted for temperature and pH play a vital role in the growth, production, and of any microbial synthesis. Temperature is one of the most important parameters most essential for the success of a fermentation reaction.The results show that highest phytase production, and fermentation temperature at 28^0C in *Hanseniaspora guilliermondii* S1 MG663578 (395±3.2) whereas *Pichia fermentans* S2 MG663579 (365±2.7) (**Figure 12**). Production started to decline after a further increase in temperature. Most of the yeast grows optimally within a wide range of pH. The pH has a profound effect on the phytase production. There was a gradual decline in increased temperature conditions respectively [22]. Similarly, there was the highest phytase production at pH and the enzyme yield was reduced with an increase in alkaline pH in *Hanseniaspora guilliermondii* S1 MG663578 (395±1.3) whereas *Pichia fermentans* S2 MG663579 (365±2.3) (**Figure 13**). There was >60% reduction

in the enzyme production at more than pH 5.5.The results were directly compared with the previously reported findings, there was a linear variation between phytase production and pH up to 9.5 for fungi and the enzyme yield was reduced with an increase in pH. This is in support of earlier reports where they have shown that pH ranging from 4.5 to 6.0 is optimum for filamentous fungi [23].

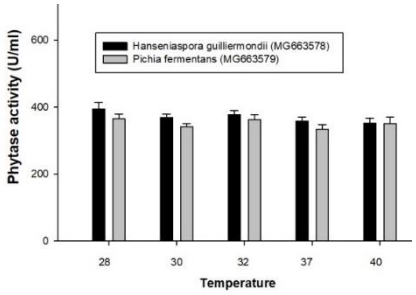

Figure 12. Different incubation temperature.

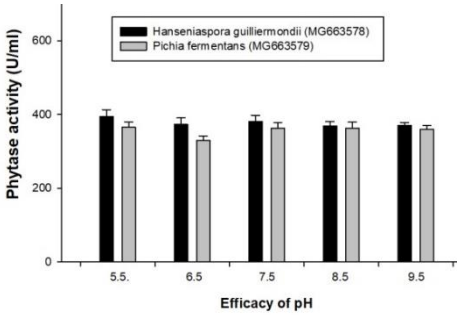

Figure 13. Different condition of pH.

Since the fermentation incubation period is crucial, it is also important to find out the optimum growth and yield for enzyme production. Some organisms are reported to yield maximally in the log phase of growth, whereas some at their stationary phase. In the present work, as maximum phytase activity by both strains was obtained at 72 h fermentation and decreased thereafter.

3.4. Enzyme kinetics.

The Lineweaver-Burk plot of [V]-1 against [S]-1 was presented in **Figure 14** the reaction to follow Michaelis–Menten kinetics thus, Km and Vmax values were found to be 3.3 mM and 0.4 µmol/min respectively using phytic acid as a substrate in *Hanseniaspora guilliermondii* S1 MG663578. These values were previously reported well within the range for microbial phytases [24-25].

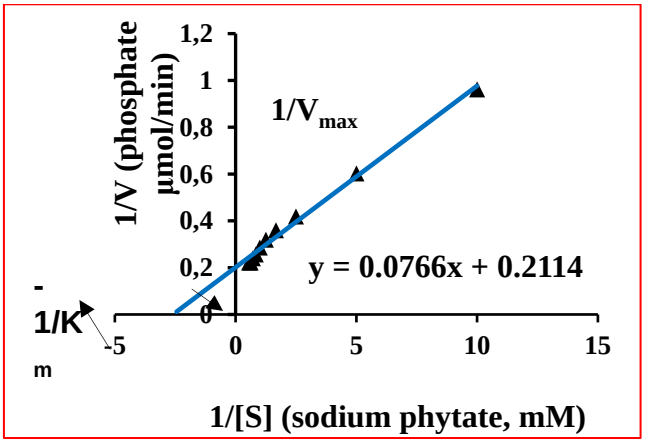

Figure 14. Lineweaver-Burk plot of phytase.

4. Conclusions

It can be concluded that accurate and constant concentration of peptone, dextrose, yeast extract,malt extract pH, temperature, and phytic acid play important role in phytase activity by *Hanseniaspora guilliermondii* S1 MG663578 and *Pichia fermentans* S2 MG663579. There are many scientists who took the help of medium optimization for solid state fermentation, with all the above analysis we can conclude that suitable for increased phytase production by these organisms. It has been found that phytase activity increased under acidic pH 5.5 maximum activity (395U/ml).

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Conflicts of Interest

The authors declare no conflict of interest.

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