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RESEARCH ARTICLE

PRODUCTION OF PHYTASE BY ACIDO-THERMOPHILIC STRAIN OF *KLEBSIELLA sp*. DB-3FJ711774.1 USING ORANGE PEEL FLOUR UNDER SUBMERGED FERMENTATION

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Abstract
Phytase producing strain of *Klebsiella sp*. DB-3 FJ711774.1 was isolated from poultry field soil. The bacteria produced extracellular phytase under submerged fermentation (Smf) using orange peel and other agro-residues as nutritive substrates. Among different substrates, orange peel flour and wheat bran (2% w/v) provide a maximal yield of production of phytase i.e., 3.15 and 2.41 U/ml respectively when Smf was carried out by using fermentative media supplemented with 0.2% sucrose, 0.1% $NH_4H_2PO_4$; inoculated with 1.5% cell suspension 24 h age and then, incubated at 50°C for 72 h. As such, there is no report of phytase production by using orange peel as substrate. The phytate biosynthesis by bacteria cultivation on medium based on orange peel flour was accompanied by the liberation of soluble phosphate without any extra supplementation of metal ions. Based on these results the use agro residues in the form of orange peel flour could be one of the best and cost effective alternatives to the costly pure phytate for industrial production of phytase using selected strain of *Klebsiella* spp.

Keywords: phytase, *Klebsiella* spp., submerged fermentation, orange peel flour

Introduction

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisdihydrogenphosphate) mixed cation salts of phytic acid, designed as phytate, are a group of organic phosphorus (P) compounds found widely in nature especially in legumes, cereals, and oilseed crops which serve as a major source of nutrients for the animals. These crops have an important constituent of phytic acid whose salt form, phytate, is present in more than 80% of the total phosphorus in cereals and legumes. In terrestrial ecosystems, they are synthesized by plants, accumulate in seeds during the ripening period and are regarded as the primary storage form of myo-inositol (an important growth factor) (Viveros *et al*., 2000), phosphorus in grains (Turner *et al*., 2002) and in pollen (Mehta *et al*., 2006).

The ruminants digest phytate with the help of phytases produced by their anaerobic ruminal microbiota. However, simple-stomached animals such as pig, poultry and fish are deficient in gastrointestinal tract phytases. So, in the context of human and animal nutrition, the following two aspects of phytate are critically important (Wodzinski and Ullah, 1996). Firstly, monogastric animals have only low levels of phytate-degrading enzymes in their digestive tracts, and since phytate itself is not resorbed, feed for animals is supplemented with inorganic phosphorus to meet phosphorous requirement; and secondly, phytate is an antinutrient constituent in plant-derived food and feed, since it form complexes with proteins, amino acids (Pallauf and Rimbach, 1997) and variety of metal ions such as calcium, magnesium, iron and zinc. It forms complex with these minerals because it poses a high phosphate content, which results in a high negative charge over a wide pH range. So, it chelates with positively charged divalent cations, rendering a poor absorption of the bound metals in small intestine (Cheryan, 1980).

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This is partially attributed to the wide-spreading human nutritional deficiencies of calcium, iron, zinc in developing countries where the staple foods are plant origin (Manary *et al*., 2002) but mineral sub-deficiencies may also occur in developed countries (Lopez *et al*., 2002). Not only with divalent minerals, phytate are also capable of binding with proteins and starch and hence create hurdle in the path of feed assimilation (Noureddini and Dang, 2009).

Because of these problems, there is considerable interest in phytate degrading enzyme. As the hydrolysis of phytate has great importance, a special class of enzymes hydrolyzing phytate has evolved – the phytases (*myo*-inositol hexakisphosphate phosphohydrolases) which hydrolyze phytate to less phosphorylated *myo*-inositol derivatives (in some cases to free *myo*-inositol), releasing inorganic phosphate and other divalent elements. Therefore, phytase in animal feed reduces the need of extra supplementation of phosphorus. As a result, fecal phosphate excretion by the animal is reduced by up to 50% (Haefner *et al*., 2005) and hence, environment is protected from excessive phosphorus runoff pollution. Phytases have been found in plants, microorganisms, and in some animal tissues (Konietzny and Greiner, 2002). The first commercial phytase products were launched into market in1991 (Haefner *et al*., 2005).

Therefore, phytases are considered to be potential candidate for use as an enzyme that have great value in enhancing the nutritional quality of phytate-rich foods and feeds (Haefner *et al.,* 2005). Phytases are not only used as animal feed additive in diets for monogastric animals but there is great potential for the use of this class of enzymes in processing and manufacturing of food for human consumption (Haefner *et al*., 2005). In addition, phytase would be an eco-friendly product, reducing the amount of phosphorus entering the environment or problems resulted by eutrophication and constant chelating of nutrient factors from soil, as supplementation of phytase in the diets for monogastric animals reduces the faecal phosphate excretion up to 50%.

Kim *et al*., (1998) had reported the phytase production by *Bacillus sp.* DS11 through Smf of crude phytate in medium containing wheat bran. Wheat bran is cheap agro residue source of phytate but still it is used as fodder but orange peel is an organic fruit waste which is a completely waste and not used anywhere so we showed more interest in upgrading this waste for some value added products along with solving the disposal problems of waste. Although citrus pulp (Spier *et al*., 2011) was used for the produce of phytase by solid state fermentation but citrus fruit was not specified. The production in orange peel medium will be highly cost-effective. Phytate content (% dry weight) in orange peel is 0.062-0.082. Although phytate content in orange peel is less but still our isolate utilizes this phytate very efficiently and giving higher production of phytase. Citrus of all species are widely grown in tropical and subtropical regions. It has been proved that a rational use of by-products allows to eliminate the myth that supplementation may be carried out only through imported cereals and that their use as food for animal production may contribute to the conservation of the environment. But when their by-product is under-utilized it causes some serious local environment pollution problems as well as due to perishable property of fruit, it would be convenient to develop methods of preservation that would enable these plant material to be utilized as animal feeds for longer periods of time (Aguilera *et al.*, 1997). Chemical composition as well as some trace elements, ascorbic acid, carotenoids dietary fiber, total polyphenols and their antiradical efficiency, using the 2,2-diphenyl-1-picrylhidracyl (DPPH) were assessed in the dried peels of orange (*Citrus sinensis*) (Rincon *et al*., 2005). Orange peel is also a good source of dietary fiber and phenolic compounds, whose use could be useful in the formulation of functional foods, taking advantage of the presence of dietary fiber and antioxidant compounds in only one ingredient. It is reported a yearly world production of 106 million tons of citrus fruits. The orange fruits represented the 63% of the world citrus production. Brazil and the United States account for 93% of world orange juice production.

Inpresent study,the production of phytase by novel strain of *Klebsiella sp*. DB-3 FJ711774.1, which utilizes phytate aerobically and produced a high yield of phytase through submerged fermentation (Smf) with orange peel flour or wheat bran was studied.

Moreover, till now there is no report of phytase production from orange peel flour and so, in this study we have exploited orange peel, one of the cheapest sources, for the very first time for extracellular production of phytases under submerged fermentation.

Materials and methods

Microorganism and culture preservation

The bacterial strain used in the present study was isolated from poultry field soil and identified as *Klebsiella sp*. DB-3 FJ711774.1 by Xcelris Labs Ltd, Ahmedabad, India and has been given National Centre for Biotechnology Information (NCBI) accession No. FJ711774.1. The pure culture was maintained by cultivation on a Nutrient Agar (NA) medium (Hi media, India) and preserved at 4^{o}C.

Raw materials used as substrates

Different substrates like wheat bran, wheat husk, rice husk, black chana covering, sugarcane bagasse lemon and orange peel flour which were exploited as nutritive substrates for submerged fermentation medium formulation, were collected from local areas, dried at 60^{o}C and powdered in a grain mill, Spectrum Industries, India. The phytate content in different substrates was followed by the method of Gulati *et al*., 2007.

Submerged fermentation for phytase production

Each of the seven substrates (2% w/v) were mixed in 250 ml Erlenmeyer flask with 25 ml of basal medium containing (g/l): NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.5; KCl,0.5; $FeSO_4$,0.01; $MnSO_4$,0.01; pH 5.5 and sterilized at 121.1^{o}C for 20 minutes. After cooling, the medium was inoculated with 2 % bacterial cell suspension of 12 h old cultures under aseptic conditions. These flasks were then incubated at 45^{o}C for 72 h under shaking conditions (120 rpm). For obtaining extracellular enzyme, the biomass was removed by centrifugation at 11000 x g for 10 minutes and the supernatant was assayed for phytase activity.

Phytase activity assay

Phytase activity was assayed in a reaction mixture containing sodium phytate (Sigma; 0.5% w/v) prepared in sodium acetate buffer (0.2 M, pH 5.5) and suitably diluted enzyme. The reaction was stopped by adding an equal volume of 15% trichloroacetic acid after 30 minutes of incubation at 50^{0}C. The liberated phosphate ions were quantified by mixing 100 µl of assay mixture with 900 µl of 1.0 M H_2SO_4, 10% ascorbic acid, 2.5% ammonium molybdate (3:1:0.1) (Gulati *et al*., 2007). After 20 minutes of incubation at 50^{0}C, absorbance was measured at wavelength of 820 nm. A control sample assay was run simultaneously that contained all the reagents without enzyme. One enzyme unit was defined as the amount of enzyme liberating 1µmol of inorganic phosphate in 1minute under the assay conditions.

The influence of some process parameters evaluation

Various optimum parameters required for maximum phytase yield biosynthesis by *Klebsiella sp*. DB-3 FJ711774.1 under Smf were determined for substrate and its concentration (0.5-5.0%), incubation temperature (30-60^{o}C), inoculums size (0.5-2.5%, v/v), pH (3.5-8.0), incubation time (24-120 h), addition of sodium phytate (0.2-1%, w/v), supplementation of carbon sources (glucose, galactose, maltose, lactose, sucrose, starch, mannitol) at 0.2%, w/v, supplementation of nitrogen sources (ammonium nitrate, ammonium sulphate, ammonium dihydrogen phosphate, beef, tryptone, urea, peptone) at 0.1%, w/v, quantitative effect of different selected nitrogen source (0.1-0.5%). All experiments were carried out in triplicate and the mean values were reported with standard deviation.

Results and discussion

Klebsiella sp. DB-3 FJ711774.1 is able to produce high yield of phytase under Smf in the medium

containing cost effective agro residues as sole source of crude phytate and carbon source.

Table 1. *Phytate concentration in different vegetal substrates*

Source	Phytate % dry weight
Wheat Bran	0.25-1.37
Wheat Husk	0.046-0.074
Rice Husk	0.072-0.099
Black chana Covering	0.029-0.072
Sugarcane bagasse	0.038-0.084
Lemon peel flour	0.062-0.082
Orange peel flour	0.062-0.082

Effect of substrate and its concentration

The phytate content in different substrates (% dry weight) was established by using the method of Gulati *et al.* (2007). The qualitative and quantitative effect of substrate on enzyme production was studied. Vegetal substrate at a concentration of 2% w/v was found to be optimum for maximum enzyme production. The phytase activity in different substrates (2%, w/v) was found to be 0.86 U/ml in wheat bran, 0.53 U/ml in wheat husk, 0.41 U/ml in rice husk, 0.49 U/ml in black chana covering, 0.67 U/ml in sugarcane bagasse, 0.73 U/ml in lemon peel flour and (0.97 U/ml) in orange peel flour (Table 1). The high phytase activity was obtained in case of wheat bran and orange peel although the maximum enzyme activity (0.97 U/ml) was reported when orange peel flour was used as substrate. The selection of a substrate for large-scale enzyme production by fermentation depends on its availability and cost. In this regard, several low cost waste agro residues were used for production of phytase by *Klebsiella sp*. DB-3 FJ711774.1 through submerged fermentation (Smf) and the best nutritive substrates for phytase production was selected i.e., orange peel flour and wheat bran. The high production of phytase in case of orange peel flour and wheat bran may be due to some inducing factors that accelerate enzyme biosynthesis. The concentration of phytate is a very important determining factor for phytase biosynthesis for most fungi, yeast and bacteria. In comparison to our results, Kim *et al*., (1998) found maximum phytase production in medium containing wheat bran. But orange peel is not exploited till now for phytase production. Phytate content in orange peel is 0.062-0.082 (% dry weight). Although the content of phytate in orange peel is not much high as compared to other substrates but still it showed maximum phytase production because in this phytate is easily available to bacterial strain as it is not in the bounded form. The actual mode of phytase induction in a particular concentration of phytate has not been explained clearly until now. Maga (1982) mentioned that higher concentration of phytate leads to chelation of divalent cations which impair the metabolism as well as growth of the organism.

Effect of cultivation time

Time course of cultivation was recorded for 120 h. The enzyme production was started after 30 h of the growth of culture and reached at the highest level at 72 h, after which it decreased. Maximum phytase activity was observed at 72 h when both substrates were used i.e. 0.97 and 1.02 U/ml in wheat bran and orange peel flour respectively (Figure 1).

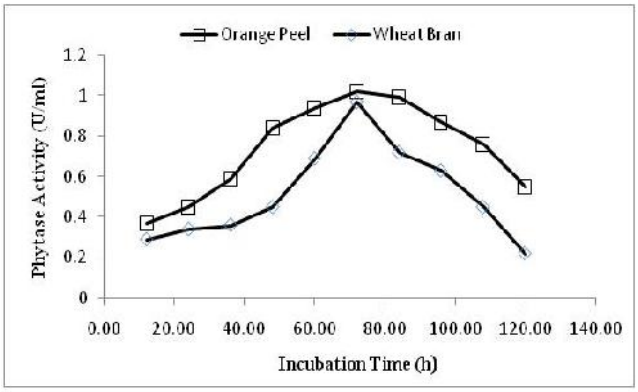

Figure 1. *Effect of cultivation time on phytase production by Klebsiella sp. DB-3 FJ711774.1 by submerged cultivation*

One advantage of this strain is that it can produce maximum enzyme yield within a short period of cultivation (72 h) as compared to reported different bacterial and fungal strains. Some workers reported maximum phytase production at short cultivation time (14 h) for *E. coli* (Kleist *et al*., 2003) and in 32 h for *Lactobacillus amylovorus B4552* (Sreeramulu *et al*., 1996).This feature makes the strain a promising candidate for production of phytase on a commercial scale. The decrease in enzyme yield after 72 h could be owing to the increased biomass production, which, in

turn, might have resulted in the depletion of carbon source and nutrients in the medium, affecting the enzyme synthesis. It could also be the result of inhibition of cells or denaturation of the enzyme caused by interaction with other compounds in the medium (Ramesh and Lonsane, 1987). The period required for cultivation for a optimum yield of enzyme production depends on the growth rate of the microorganism and on the regulation mechanism of biosynthesis of particular enzymes.

Effect of temperature of cultivation

The effect of temperature varying between 30^{0}C and 60^{0}C on production of phytase was studied. Maximum phytase activity was observed at 50^{0}C by using both the substrates i.e., 1.50 U/ml on wheat bran and 1.85 U/ml on orange peel flour (Figure 2). Similar to our observation, some of bacterial strains such as *Mitsuokella jalaludinii* and *Escherichia coli* have been reported to produce phytase between at 37-39^{0}C, respectively (Lan *et al.*, 2002). Temperature affects various metabolic processes such as protein denaturation, enzymatic inhibition, promotion or inhibition on production of a particular metabolite, cell death etc. For food digestion in monogastric animal's stomach, enzyme has to be thermo-tolerant so that it can withstand high temperature during pelleting process of food.

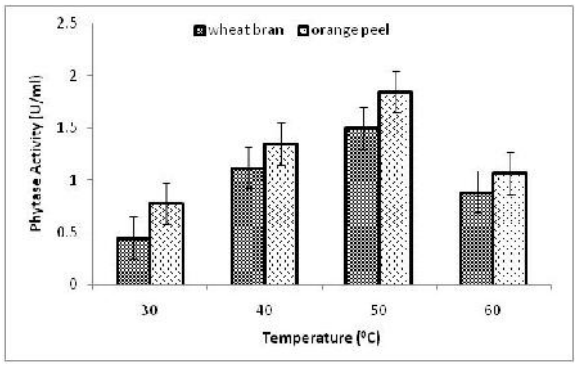

Figure 2. *Effect of temperature on phytase production by Klebsiella sp. DB-3 FJ711774.1 by submerged fermentation*

Effect of inoculum size

The size of inoculum plays an important role in the production of metabolites under submerged fermentation. The effect of inoculum size (0.5-2.5%) on phytase production by *Klebsiella sp.* DB-3 FJ711774.1 under submerged fermentation was evaluated. It was found that 1.5% (v/v) inoculum resulted in maximum phytase activity in both substrates (Figure 3) i.e., 1.62 U/ml in wheat bran and 1.97 U/ml in orange peel flour. Lower level of inoculum may not be sufficient for the growth and enzyme synthesis on different substrates as increased number of cells ensures a rapid proliferation of biomass and enzyme biosynthesis.

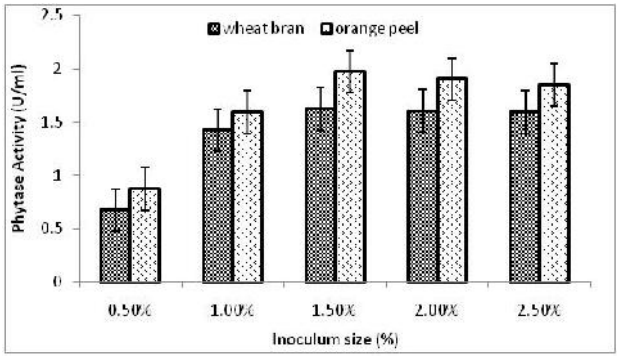

Figure 3. *Effect of inoculum size on phytase production by Klebsiella sp. DB-3 FJ711774.1 by submerged fermentation*

Above 1.5 % inoculum, the decrease in the phytase production may be due to depletion of nutrients as a result of enhanced biomass production thereby decreasing the metabolic activity. In the literature, growth and related phytase production has been reported in various bacterial and fungal species. Kammoun *et al.*, 2011, reported growth related phytase production in *Bacillus subtilis* US417. Phytase yield and biomass formation were strongly correlated with the inoculum age, indicating strong growth associated phytase production by selected fungal strains *Mucor racemosus, Rhizopus oligosporus*, *Aspergillus niger* (Roopesh *et al.*, 2005; Sabu *et al.*, 2002; Krishna *et al.*, 2001).

Effect of pH

Various range of pH (3.5-8.0) was tested in order to enhance phytase yield. The studied bacterial strain gave maximum activity i.e., 1.89 U/ml by cultivation on wheat bran at pH 5.0 and 2.47 U/ml on orange peel flour at pH 5.5. The optimal pH for phytase production for most bacteria and fungi is in the range of 5.0 and 7.0. There is no report of phytase production at alkaline pH. Lan *et al.* (2002) reported that phytase production and bacterial growth were significantly inhibited when

pH level of the medium was lower than 6.8. Vats and Banerjee (2004) studied the growth and production of phytase by a filamentous fungus, *Aspergillus niger* va Teighem, in submerged culture under uncontrolled pH conditions. However, maximum of phytase was obtained when the broth pH was maintained at pH 5.5. *Klebsiella sp*. DB-3 FJ711774.1 produces phytase in acidic medium at high temperature of 45^0C by using wheat bran (pH 5.0) or orange peel flour (pH 5.5) as carbon sources. This feature makes the strain to be active in thermo-acidic environment occurring in stomach having acidic pH. Therefore, this enzyme can find application in animal feed industry for improving the nutritional status of feed.

Effect of phytate supplementation

The effect of phytate on phytase yield was studied by adding different concentration of phytate (0.2-1%) in the fermentative medium. No inductive effect was observed at different concentration of the phytate. Supplementation of phytate was found to decrease the enzymatic activity (Figure 4). This may be due to the fact that higher concentration of phytate leads to the formation of non-reversible bonds with surface proteins and impair the metabolism as well as growth of the organism. One of the most striking observations in this present investigation is that the highest enzyme production was obtained when wheat bran (1.90 U/ml) or orange peel flour (2.48 U/ml) were used as phytate substrate as compared to the medium containing only pure phytate (1.50 U/ml). This observation makes wheat bran or orange peel flour as one of the best as well as cheaper substrate alternative to the costly pure phytate for industrial production of microbial phytase.

Many workers reported also that phytate had no influence on the synthesis of phytase in *Escherichia coli*, but in *Klebsiella terrigena*, phytase was produced only in the presence of phytate. In the case of *Bacillus subtilis*, minimal medium containing inorganic phosphate and phytate did not induce phytase production, but a defined medium in which phytate was the sole source of phosphate, phytase production was induced (Kerovuo *et al*.,1998).

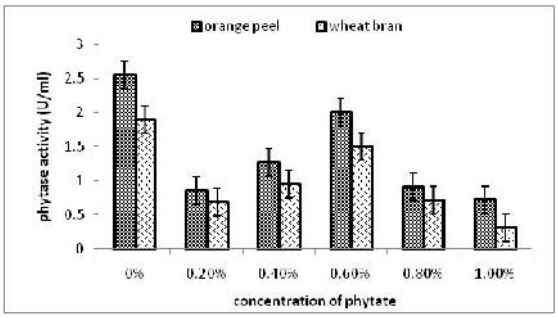

Figure 4. *Effect of supplementation of different concentration of phytate on phytase production. Data are given as means ± SD, n=2. (Fermentative conditions: cultivation on basal medium containing 2% (w/v) orange peel flour or wheat bran as substrates, pH 5.5, temperature of 45^0C, for 72 h)*

Effect of carbon sources

Effect of different carbon sources (0.2 % w/v) on the production of phytase was evaluated (Figure 5).

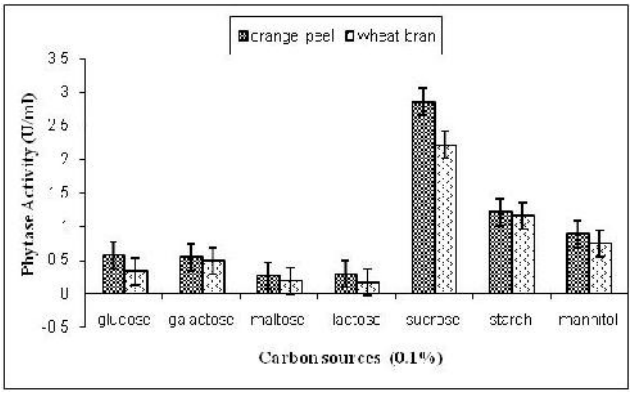

Figure 5. *Effect of carbon sources on phytase production by Klebsiella sp. DB-3 FJ711774.1. Data are given as means ± SD, n=2. (Fermentative conditions: cultivation on basal medium containing 2% (w/v) orange peel flour or wheat bran as substrates, pH 5.5, temperature of 45^0C, for 72 h)*

The results showed that *Klebsiella sp*. DB-3 FJ711774.1 gave maximum yield of phytase by added sucrose i.e., 2.21 U/ml in medium with wheat bran and 2.85 U/ml in medium with orange peel flour. All other carbon sources suppressed the phytase yield in presence of both wheat bran and orange peel flour. Glucose (1% w/v) was found to be optimal for phytase production by *Enterobacter sp*. 4 (Yoon *et al.*, 1996), *Lactobacillus amylovorus B4552* (Sreeramulu *et al*., 1996). Glucose at 2% (w/v) concentration was used for maximum phytase production by *Bacillus subtilis*

(Kerovuo *et al*., 1998). Wheat bran (6%) was a good carbon source for phytase production by *Bacillus sp*. DS11 (Kim *et al*., 1998). *Mitsuokella jalaludinii* (rumen bacteria) and *Bacillus laevolacticus* produced phytase optimally in the presence of rice bran and pea flour, respectively (Gulati *et al*., 2007; Lan *et al*., 2002).The response of different strains for phytase production varies with the nature and concentration of carbon source in medium.

Effect of nitrogen sources

Nitrogen source is also very essential component for growth and enzyme production by the microorganisms. The influence of different nitrogen sources (0.1% w/v) on phytase production was evaluated. The results revealed that ammonium dihydrogen phosphate ($NH_4H_2PO_4$) showed stimulatory effects added in medium with containing wheat bran or orange peel flour. Maximum yields of phytase production was obtained by supplementation with 0.1% $NH_4H_2PO_4$ of basal media i.e., 2.41 U/ml medium with wheat bran and 3.15 U/ml medium with orange peel flour (Figure 6).

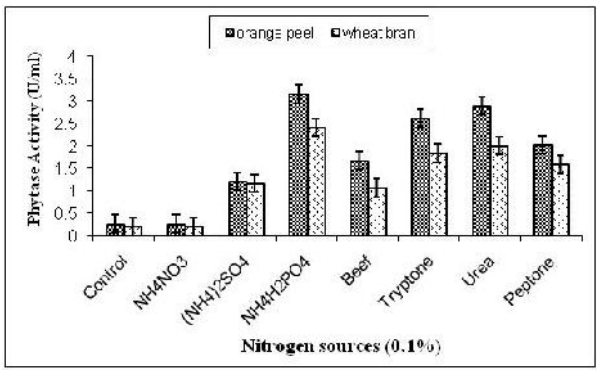

Figure 6. *Effect of nitrogen sources time on phytase production by Klebsiella sp. DB-3 FJ711774.1. Data are given as means ± SD, n=2. (Fermentative conditions: cultivation on basal medium containing 2% (w/v) orange peel flour or wheat bran as substrates, pH 5.5, temperature of 45^{0}C, for 72 h)*

Different workers reported different inorganic nitrogen sources for optimum phytase production in fungi and bacteria. Bogar and coworkers (2003) found that urea and ammonium sulphate were better than ammonium nitrate and other natural nitrogen sources for phytase production by *Aspergillus ficuum*, however, for *Mucor racemosus* a combination of casein and ammonium sulphate was a better choice. On the contrary, supplementation of the fermentation medium contains oil cake with 0.5 % ammonium nitrate increased phytase activity at *Rhizopus spp*. (Ramchandran *et al*., 2005) and by optimization of rice bran and soybean meal concentration, the phytase production by *Mitsuokella jalaludinii* increased by about 2.6 times (Lan *et al*., 2002). Phytase biosynthesis yield at *Pichia anamola* increased when beef extract in the medium was replaced with peptone (Vohra and Satyanarayana, 2001). The reports in the literature suggest the positive influence of addition of inorganic and organic nitrogen sources for the production of phytase.

Effect of metal ions

Phytases from different microorganisms differed in their requirement for metal ions for their activity. Hence, it is important to know the kind of ions and their concentration in achieving maximal phytase efficiency and stability. The effect of different divalent cations (5 mM) such as sulphates of Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} and chlorides of Ca^{2+}, Na^{+}, K^{+}, Ba^{2+} on phytase production was evaluated. The results showed that phytase yield was suppressed due to the presence of additional metal ions in media with orange peel flour or wheat bran as carbon sources (Figure 7). Hence, it further reduces the production cost of enzyme.

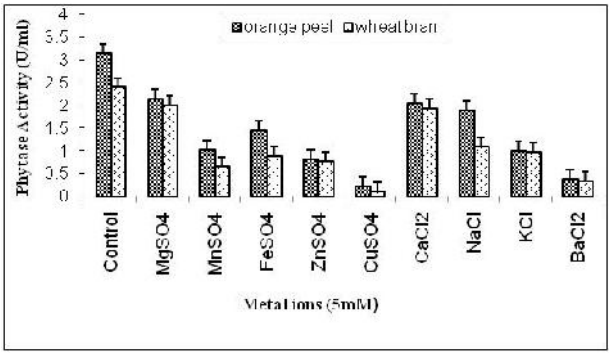

Figure 7. *Effect of metal ions on phytase production by Klebsiella sp. DB-3 FJ711774.1. Data are given as means ± SD, n=2. (Fermentative conditions: cultivation on basal medium containing 2% (w/v) orange peel flour or wheat bran as substrates, pH 5.5, temperature of 45^{0}C, for 72 h)*

There are many such reports on the effect of metal ions on phytase production by bacteria and fungi. Phytase of *Saccharomyces castellii* was slightly inhibited in the presence of 5 mM Ca^{2+}, Mg^{2+},Mn^{2+} and Fe^{2+}. The cations Zn^{2+} and Cu^{2+} (0.5 mM) caused around 50% inhibition of enzyme activity (Segueilha *et al*., 1992). *Bacillus subtilis* phytase required Ca^{2+} for preserve its active conformation (Kerovuo *et al*., 1998).

Conclusions

In this study the first time the exploitation of orange peel flour for the production of phytases from bacterial source i.e., *Klebsiella sp*. DB-3 FJ711774.1 was evaluated. By using cheaper and easily available substrates and use of tap water as moistening agent, the lower down the input cost for enzyme production is one of the reason that limit the use of phytase at the industrial level. Based on the present study, it is concluded that novel strain of *Klebsiella sp*. DB-3 FJ711774.1 offers scope for the industrial production of phytase under submerged fermentation using cost effective agro residues. The additional fermentative conditions as qualitative effect of some carbon and nitrogen sources, pH and temperature of cultivation, time of cultivation, inoculum size and the effect of supplementation of medium with phytate or metal ions were studied and optimized. The optimum conditions for phytase production varying with nature of principal carbon sources, wheat flour or orange pee l flour. This condition are cultivation time of 72 h, 50^0C incubation temperature, 1.5% inoculums size for both the substrates but optimum pH varies for both the substrates i.e., pH 5.0 for wheat bran and pH 5.5 for orange peel flour. Optimum carbon and nitrogen sources concentration are also same for both the substrates.

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