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ORIGINAL ARTICLE

Optimized Production of Pectate Lyase by a Thermoalkaliphilic Strain of *Bacillus licheniformis*

Sneha A, Sagar S, Meenakshi C, Rashmi R
Dept. of Biochemistry, Center for PG Studies, Jain University, Bangalore, India
18/3, 9th Main, 3rd Block, Jayanagar, Bangalore-560011
Corresponding author email: rashmikrishna1@yahoo.co.in

ABSTRACT
Pectate lyases are a class of pectinases that have a multitude of applications in various processes. Of late, there is extensive research for identification of organisms with desirable stable characteristics that can be used for enzyme production. The best sources for these are extremophilic environments including hot water springs. A thermoalkaliphilic strain of Bacillus licheniformis has been isolated from the hot water springs of North India. Common agricultural and industrial wastes were used as carbon sources and the medium for pectate lyase production was optimized with various nitrogen sources and inducers as well. The best identified components were used in different combinations for maximum enzyme production. There was six fold increases in pectate lyase production as a result of this optimization.
Key words: *Pectate lyase, extermophile, thermoalkaliphilic, growth curve, medium optimization, Bacillus licheniformis, hot water springs*

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INTRODUCTION

Pectinases is a collective term for the group of enzymes that breakdown pectin. They are extracellular metabolites produced by different organisms and have a plethora of applications in industrial processes. They amount to 25% of the global sales of food enzymes [1]. Pectate lyase is the first of the pectinases that acts on encountering the substrate, pectin. They perform non-hydrolytic breakdown of pectin, characterized by a trans-eliminative split of the pectic polymer producing a 4, 5 unsaturated product. They have applications in paper, textile, and food processing as also in brewing. The exhaustive list of their applications has been compiled by Jayani *et al*. [2].

Almost all bacteria and fungi that are capable of growing on pectinaceous substances produce PLs, either as the major enzyme or as an enzyme cocktail with other pectinases. Driven by increasing industrial demands for biocatalysts that can cope with industrial process conditions, considerable efforts have been devoted for the search of sturdier enzymes. Enzymes for use in commercial applications with desirable biochemical and physico-chemical characteristics that can be produced at lower cost, have been the focus of much research. Enzymes obtained from mesophilic organisms have restricted application in industry because of their limited stability to extremes of temperature, pH and ionic strength. Extremophiles, especially thermophiles and alkaliphiles, are a source of extremozymes which are not only stable but also optimally active under extreme conditions. Given their robust nature, extremozymes can be subjected to harsh environments, including elevated temperature, high pH, surfactants, bleach chemicals and chelating agents. Many thermophilic and/or alkaliphilic enzymes that are being used commercially include lipases and cellulases in laundry and dishwashing detergents, PLs in degumming of fibres, proteases for de-hairing of hides and skins, improving smoothness and dye affinity of wool [3].

Microbial diversity in hot water springs has been persistently researched upon for identifying such enzyme producers. Not many such reports are available from India though it is host to innumerable hot water springs. Bacteria are the most prominent producers of extremozymes. Many species of Bacilli have

been reported to produce alkaline PLs [1]. Use of alkaline PLs of Bacillus in waste water treatment [4], bioscouring [5], retting and degumming of fibers [6, 7] and during cocoa fermentation has been reported [8]. The present work reports the optimization of PL production from a strain of *Bacillus licheniformis* isolated from hot water springs.

MATERIALS AND METHODS

Chemicals

Polygalacturonic acid was purchased from Sigma Chemicals, USA and pectin from HiMedia Chemicals Ltd., Mumbai, India. All other chemicals were of analytical grade. Bacterial identification was done by Chromous Biotech Pvt. Ltd., Bangalore, India. Collection of samples and isolation has been done between in July-Dec 2007. Further work has been done during the year 2011-13.

Preliminary Screening

Water and soil samples from hot water springs located in Gangotri, Yamunotri, Badrinath and Kedarnath, Uttarakhand, India were collected in sterile wide-mouthed glass bottles and transported to laboratory where they were maintained at 37°C until use. Isolation was carried out by using Luria Bertini (LB) medium [9] after amending it with 0.5% pectin (as the sole source of carbon) and 0.25 g/L magnesium sulphate, pH 9.0. Water samples were serially diluted and enriched in the above broth by incubating at 55°C. Soil samples (0.1g) were suspended in 1.0ml saline, serially diluted and enriched as above. Aliquots (0.1ml) of various dilutions of the enriched broths were spread on agar plates of the above medium and incubated at 55°C until visible colonies appeared. The colonies were replica plated and used for screening [10]. Pectinase producing colonies were identified by flooding with Gram's Iodine to visualize clear zone of pectin hydrolysis and compared by measuring the enzymatic index (EI), given as the ratio of the diameter of zone of clearance to that of the diameter of colony.

Secondary Screening

Three isolates with higher EI were grown in 50mL of the above broth for 3 days at 37ºC, 150 rpm. The culture filtrate was then obtained by separating the cells by centrifugation and used to assay for PL activity. The isolate showing highest activity was chosen for further studies.

Characterization of the Isolate

Isolate M9, which showed maximum PL activity, was identified by standard biochemical tests and 16s rRNA sequencing. To identify the extremophilic characteristics of the isolate, it was subjected to growth at various pH and temperature. Optimum growth conditions were identified by incubating 10ml of inoculated broth (pH 9.0) at 37, 45, 50, 55, 60, 70, 80 and 90°C and also by adjusting the pH of broth to 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. After 24h, growth was monitored by measuring absorbance at 600nm [10]. The effect of pH and temperature was analysed by one way ANOVA with the help of SPSS software.

Heat resistance was determined by incubating 10ml of inoculated broth at 100°C. After different time intervals (10 to 80min), the heat treated broth was streaked onto agar plates, incubated at 37°C and observed for recovery of growth. Specific growth rate, defined as the rate of growth per unit amount of biomass of M9 on pectin was determined by the method of Madigan *et al*. [11].

Optimization of Production Medium

To identify a better basal medium for pectate lyase production, two different medium were tested: Luria Bertani Medium (LB) and Mineral Pectin 7 Medium (MP7). The composition of LB medium (in g/L) was as follows: Tryptone-10.0, Yeast extract-5.0, NaCl-5.0, Pectin-10.0, $MgSO_4$-0.25, pH-8.5. The composition of MP7 medium is as given below. Basal medium (g/500ml): Pectin-5.0, Yeast extract-1.0, Na_2HPO_4-6.0, KH_2PO_4- 4.0, $(NH_4)_2SO_4$- 2.0. Mineral solution (1ml of the respective stock solutions were added per 500ml): $FeSO_4$-0.1%, $MgSO_4.7H_2O$-20%, $CaCl_2.2H_2O$-0.1%, H_3BO_3-0.001%, $MnSO_4.H_2O$-0.001%, $ZnSO_4.7H_2O$-0.007%, $CuSO_4.5H_2O$-0.005%. Basal medium (500ml) and mineral solution (500 ml) were mixed thoroughly and pH was adjusted to 8.5. The medium showing better activity was chosen for further optimization.

Submerged fermentation was carried out in 250ml Erlenmeyer flasks containing 50mL of the chosen medium for 7 days in a shaker incubator (150 rpm). Aliquots of the fermentation broth were removed at intervals of 24h, centrifuged at 10,000g at 4°C and the supernatant used as enzyme source.

Different carbon and nitrogen sources were incorporated into the chosen basal medium for enhancement of PL activity. Commonly generated wastes like orange peel, lemon peel, lentil peel, carrot peel, banana peel, apple pomace, orange pomace, bagasse, saw dust and coir pith were used as sources of carbon. Organic nitrogen sources like peptone, yeast extract, urea and inorganic sources like dihydrogen ammonium phosphate, sodium nitrate and ammonium sulphate were incorporated at 0.6% (w/v). Inducers like sodium dodecyl sulphate, tween 80, triton X-100, potassium chloride, mercuric chloride and manganous sulphate were added to the medium to enhance production of the enzyme.

Enzyme Assay and Protein Estimation

Pectate lyase activity was determined spectrophotometrically by measuring the formation of unsaturated products from polygalacturonic acid [12]. One unit of enzyme activity was defined as micromoles of unsaturated product liberated per ml of the enzyme in one minute. Protein content was determined by the dye binding method of Bradford [13] using BSA as standard.

RESULTS AND DISCUSSION

Isolation and Identification of Pectinolytic Bacteria

Five water samples and 4 soil samples were collected from hot water springs. Fifteen isolates were obtained and screened for pectinase production. Based on zone of clearance, EI was calculated and found to range between 1.7-5.0. Secondary screening of the three strains that showed highest EI (M9, M10 and M11) indicated that M9 has highest PL activity in the fermentation broth (grown at 37°C for 3 days). This was chosen for further studies.

Preliminary identification of M9 showed that it was motile, gram-positive rod, ~3µm in size with terminal or sub-terminal spores. It could hydrolyse starch and ferment glucose, maltose, arabinose and mannitol but not lactose and sucrose. The phylogenetic analysis using 16S rDNA sequence, showed that strain M9 had highest homology (99.9%) with *Bacillus licheniformis*. The alignment matrix and phylogenetic tree are given below (Fig. 1). PL production from isolates obtained from hot water springs includes reports from Thailand [14] and Russia [15]. However, pectinase producers isolated from hot springs of North India have not been reported so far.

Characterization of Isolate

Results of one-way ANOVA obtained for effect of temperature and pH on growth of *B. licheniformis* on pectin was found to be statistically significant at $P \leq 0.05$. The optimum growth temperature and pH was found to be 70ºC and 9.5 respectively. The isolate was tolerant to 100ºC for over an hour. Hence, the isolate of *B. licheniformis* obtained in this study is thermo-alkaliphilic. Thermoalkaliphilic strains are greatly preferred for industrial purposes owing to their ability to produce stabler enzymes. Alkaliphilic nature of *B. licheniformis* has made it a preferred source of alkali tolerant proteases that are now widely used in detergents. The thermoalkaliphilic nature of the isolate from this study may endow stable characteristics to the PL so produced.

The growth curve of *B. licheniformis* when grown in pectin containing broth (Fig. 2) showed a lag phase which lasted for 1.5h after which steady increase in growth was observed till 3h. Decline in growth was seen after a prolonged stationary phase lasting nearly 38h. The specific growth rate (µ), which is characteristic of an organisms' growth on different substrates at different growth conditions, was found to be $1.99h^{-1}$ and generation time was 4.32h.

Optimization of Pectate Lyase Production

Submerged fermentation was attempted for production of PL because of the ease of handling and down-stream processing. The basal medium had 5.0 µmoles/min/ml activity. Maximum enzyme production was in MP7 medium on the 4^{th} day of fermentation (Fig. 3). A comparative assessment of growth rate and optimum fermentation time indicated that the enzyme was produced as a non-growth associated metabolite. Martinez-Trujillo *et al*. [16] have shown that inducible pectinases are indeed produced unrelated to growth kinetics.

Of the 10 carbon sources investigated, eight substrates showed PL activity in the broth. Apple pomace and lentil peel showed 2.35 and 1.62 fold increase in comparison with basal medium (Fig. 4). Best nitrogen source was ammonium sulphate (Fig. 5). Among the inducers, mercuric chloride, potassium chloride and manganous sulphate enhanced PL activity (Fig. 6).

Agro-industrial wastes are the simplest alternatives to replace pure substrates and other costly constituents of fermentation media. Various substrates that have been used for PL production are sugarcane bagasse, orange bagasse, wheat straw, rice straw, rice bran, sawdust, wheat bran, corn cobs, coconut coir pith, banana waste, tea waste, sugar beet pulp, apple pomace, orange peel, cocoa beans [8, 17, 18]. Addition of nitrogen sources in the production media improves yield of PLs. Ammonium sulphate, peptone, yeast extract, soya bean meal have been reported to positively influence production of pectate lyase [14, 19, 20].

Enhanced production of pectate/pectin lyase by the use of Tween has been reported by Ferreira et al. [21], Batool et al. [22] among others. Similarly different supplements like SDS, Triton-X 100, EDTA, zinc sulphate and magnesium chloride have also been used to maximize production of pectinases by different organisms [22, 23]. Surfactants increase the penetration of water and increase the available surface area thereby improving enzyme production [24].

The best of the carbon, nitrogen and inducer sources were added in different combinations to the basal medium in order to obtain maximum activity.

Optimized medium 1- Apple pomace + $(NH_4)_2SO_4$ + $MnSO_4$

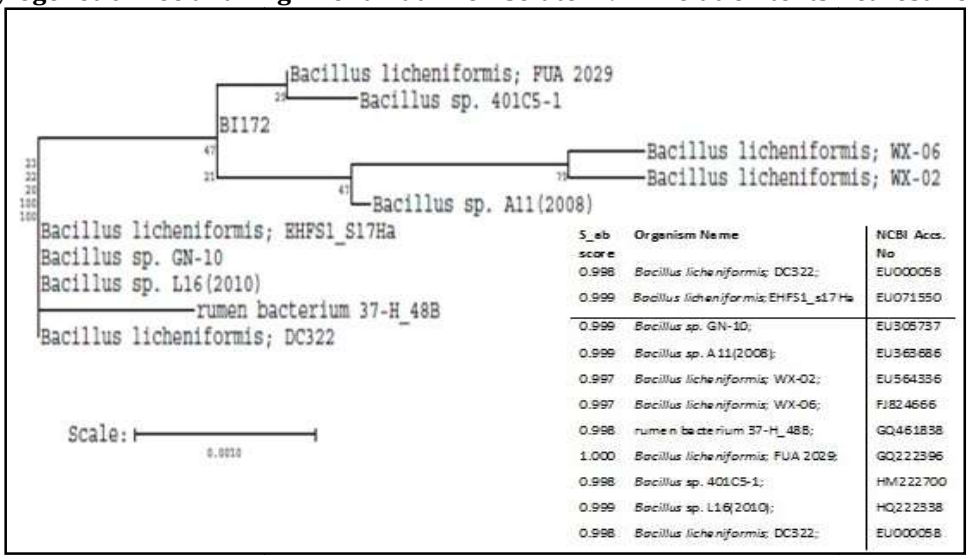
Optimized medium 2- Apple pomace + $(NH_4)_2SO_4$+ $HgCl_2$

Optimized medium 3- Lentil peel + $(NH_4)_2SO_4$ + $MnSO_4$

Optimized medium 4- Lentil peel + $(NH_4)_2SO_4$ + $HgCl_2$

Optimized medium 1 & 4 gave maximum improvement in PL activity. In comparison with the basal medium, there was 5.9 and 6 fold increase respectively (Fig. 7). It can be observed that in combination with apple pomace, manganous sulphate gave better activity while with lentil peel, mercuric chloride enhanced activity. This shows that individually each component of the medium exerts different effects and synergistically the effect can be varied due to interaction between parameters.

Fig. 1: Phylogenetic Tree and Alignment matrix of Isolate M9 in Relation to its Nearest Neighbours.



S_eb score	Organism Name	NCBI Accs. No
0.998	*Bacillus licheniformis*; DC322;	EU000058
0.999	*Bacillus licheniformis*; EHFS1_s17Ha	EU071550
0.999	*Bacillus sp.* GN-10;	EU305737
0.999	*Bacillus sp.* A11(2008);	EU368686
0.997	*Bacillus licheniformis*; WX-02;	EU564336
0.997	*Bacillus licheniformis*; WX-06;	FJ824666
0.998	rumen bacterium 37-H_48B;	GQ461838
1.000	*Bacillus licheniformis*; FUA 2029;	GQ222396
0.998	*Bacillus sp.* 401C5-1;	HM222700
0.999	*Bacillus sp.* L16(2010);	HQ222338
0.998	*Bacillus licheniformis*; DC322;	EU000058

Fig. 2: Specific Growth Curve of *B. licheniformis* on Pectin

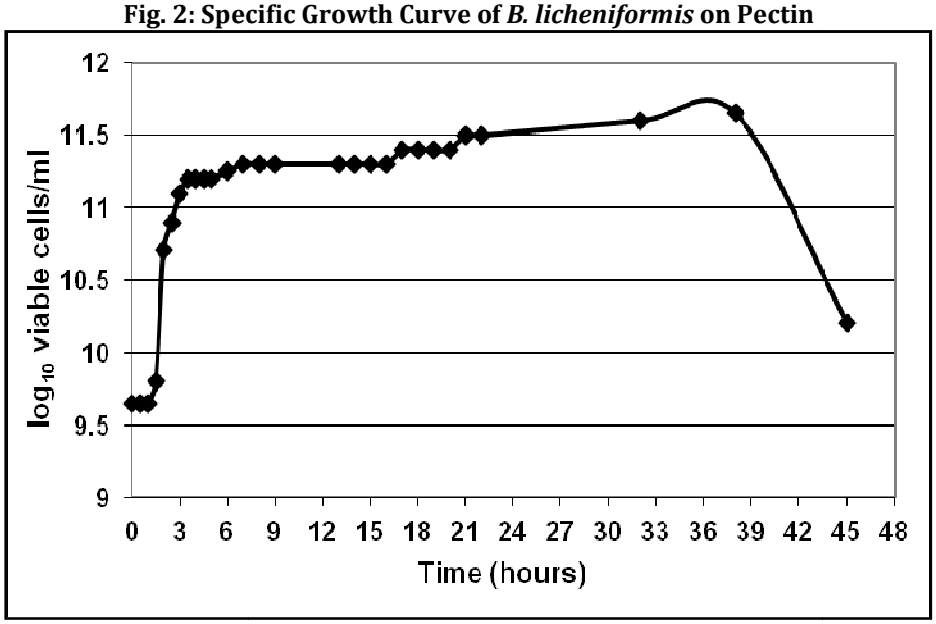


Fig. 3: Effect of Medium Composition on PL Production

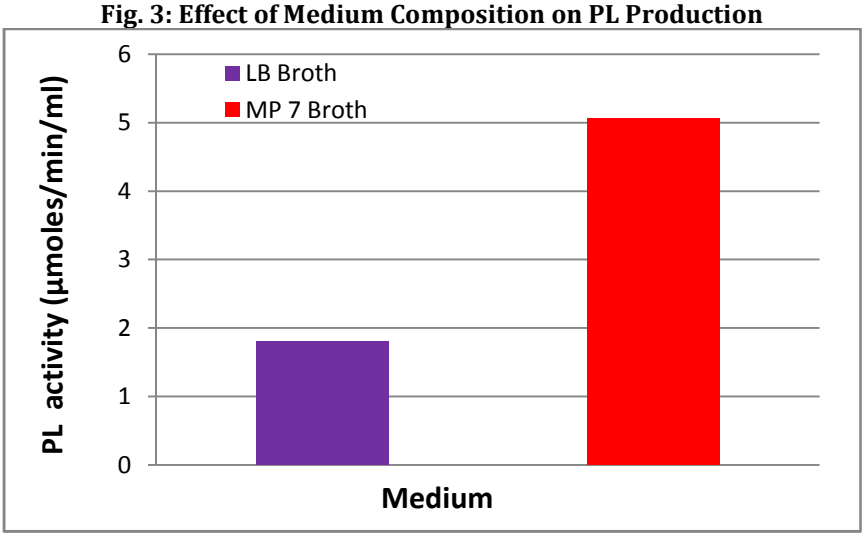


Fig. 4: Effect of Carbon Sources on PL Production

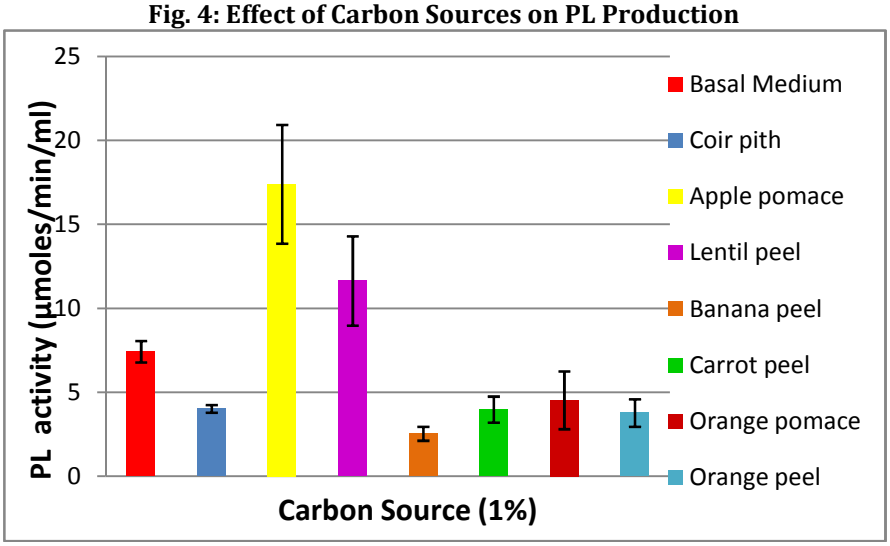


Fig. 5: Effect of Nitrogen Sources on PL Production

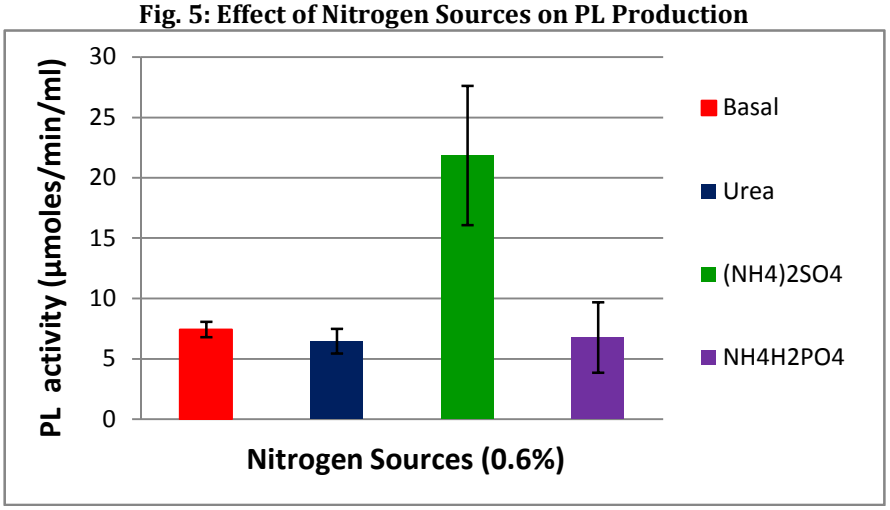


Fig. 6: Effect of Inducers on PL Production

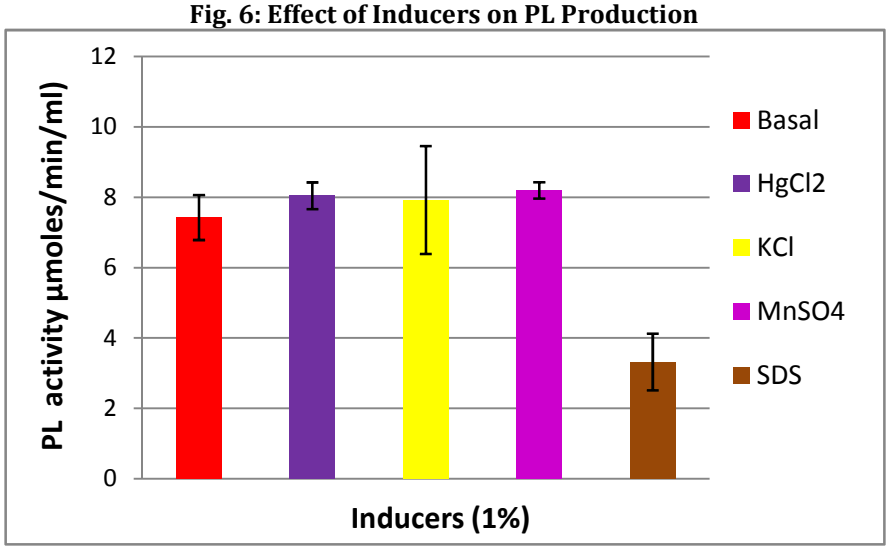
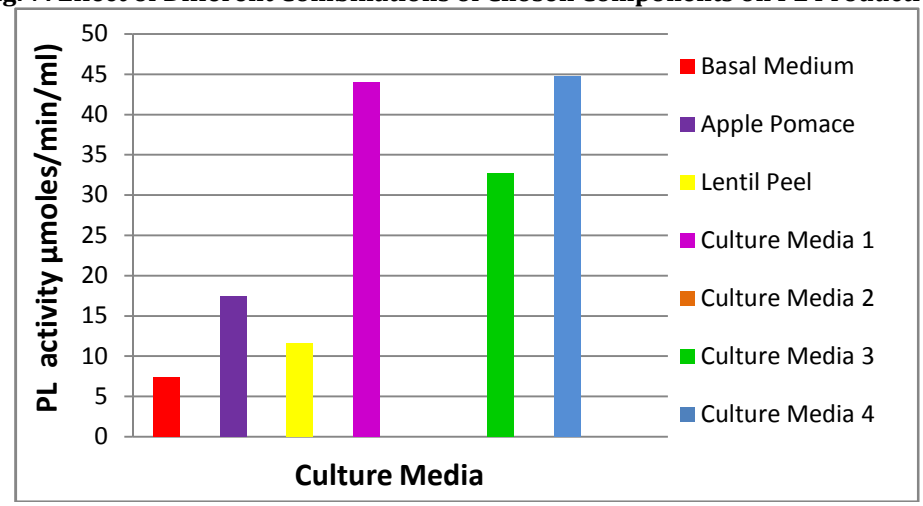


Fig. 7: Effect of Different Combinations of Chosen Components on PL Production



A combination of lemon peel powder as carbon source, yeast extract as nitrogen source and galactose as inducer has been reported to significantly increase PL production [25]. Palaniyappan *et al*. [26] reported increased PL production using a combination of wheat flour, glucose and ammonium nitrate in the production medium. A combination of bean cake powder, $MgSO_4$ and $FeSO_4$ in the fermentation medium has shown enhancement in the production of PL [19, 26].

CONCLUSION

In this study, two strategies have been investigated for production of pectate lyases. One is the identification of an industrially viable strain for use in production. The other is the use of wastes as substrates to reduce the cost of production of the enzyme. Use of both these approaches led to isolation of a thermoalkaliphilic isolate of *Bacillus licheniformis* capable of producing pectate lyase and led to six fold increase in its production using one-factor-at-a-time method of optimization.

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