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Effective Diffusivity Coefficients for Degradation of Pectin in Guava (*Psidium guajava L.*) Pulps Using Immobilized Pectinase

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Abstract

Background and objective: Recently, use of immobilization technology through cell entrapment to entrap biocatalysts has been shown as an economical method since it offers several advantages over suspension cultures such as reusability, high cell concentrations over a period of time, elimination of costly processes of cell recovery and cell recycling and simplifying the downstream processing. Understanding diffusion and permeability coefficients is necessary for the creation of optimized encapsulation systems. The major objectives of the present study included degradation studies on pectin using batch systems using soluble and immobilized pectinase enzymes.

Materials and methods: Commercial pectinase enzymes were immobilized using entrapment method for liquefaction of Guava (*Psidium guajava L.*) pulps with a wide range of applications in food industries. Guava fruit pulps were liquefied using free and immobilized pectinase enzymes to assess the intra-particle mass transfer resistance.

Results and conclusion: In the current study, effects of mass transfer on liquefaction process were revealed. Effectiveness factors for the various sizes of immobilized beads included less than 1.0, which indicated that the pectin degradation was a diffusion-controlled process. Effectiveness factors included 0.520 and 0.268 for beads having smallest and largest diameters, respectively. Intra-particle mass transfer resistance was assessed by calculating Thiele modulus (ϕ) using effectiveness factors calculated for various bead sizes. These results were further used to calculate the effective diffusivity coefficients (D_e) of guava pulps into the pores. Thiele modulus values (ϕ) were much higher than 1, which showed that the reaction was very fast and the system was mass transfer controlled. Beads with lower diameters included higher diffusivity coefficients showing a better rate of diffusion of substrates from bulk solutions.

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1. Introduction

Various post-harvest technologies have been developed for the processing of fruits to make value-added products such as jams, jellies, fruit juices and squashes. Fruit juices are much preferred for their nutritional benefits. Therefore, liquefaction of fruits using enzymatic processes is an effectively alternative method, particularly for pulpy fruits such as banana, mango and guava. Little relative studies were carried out on guava fruit pulps using free enzymes. However, no detailed studies were carried out on guava fruit pulps using immobilized enzymes.

Fruit pulps contain fruit juices, fibers, vitamins, minerals and other nutrients with water as well as pectin. Pectin in fruits and vegetables creates a big challenge for the food technologists in processing pulps, where the viscosity increases with increased pectin substances. To produce clear fruit juices, pectin break is needed to release fruit juices from the fiber contents. This technique is known as liquefaction of fruit pulps. Once clear fruit juices are available, they can be processed thermally to produce RTS beverages. Juice yield, clarity and stability can be improved by addition of enzymes as enzymes play essential roles during the processing. Various combinations of enzymes such as Pectinase 62 l and Macer8™ FJ with pectinases and other carbohydrate-degrading enzymes can be used. Guava is an important fruit because of its high nutritive values, good flavor and pleasant aroma with high consumption quantities globally. To use guava fruits through the year, preparation of guava juices is considered as the most promising method [1].

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In vegetable and fruit processing industries, commercial pectinases such as polygalacturonase, pectin methyl esterase and pectin lyase are used to separate juices from the fruit cells, clarify juices and enhance yield and quality of the juices by breaking the naturally occurring starches and pectin linkages. These starches and pectin linkages may result in undesired viscosities, poor filtrations and cloudy appearances [2]. The optimal conditions for the use of these enzymes in fruit processing include temperatures below 50°C and pH between 4 and 5.

Recovery and reuse of these enzymes are good ideas since costs of pectinase production are major contributing factors for the processing economics. One of the predicted methodologies includes immobilization of the enzymes; hence enzymes can be repeatedly used [3]. Immobilization of enzymes helps avoid extraneous compounds in final products. Furthermore, immobilized enzymes provide natural conditions similar to those in the cells [4]. These enzymes preserve their activity longer than those enzymes in solutions do. These characteristics make immobilized enzymes attractive when large throughputs of the substrates are needed and/or when enzymes are expensive. Therefore, immobilization is overall an excellent method, except that the additional mass transfer resistance problems are introduced. This occurs due to the inclusion of enzymes in polymer matrices or constrained particle sizes of the immobilized enzymes. Effective diffusivity coefficients of the substrates and intrinsic kinetic parameters of the immobilized cells are necessary for the proper design of bioreactors, which increase advantageous features of the immobilized enzymes. The effective diffusivity coefficient data of the reacting substances are important for the quantitative analysis of the bioprocesses using immobilized enzymes. A method for the calculation of substrate effective diffusivity coefficient in a particle has been described, including studies on kinetics of biodegradation for phenol and diffusion and biodegradation of calcium-alginate immobilized *Pseudomonas putida* beads with various particle sizes in a batch process [5,6]. Assuming first-order kinetics, the phenol effective diffusivity coefficient in immobilized beads was determined. An immobilized mixed culture of bacteria (*Aeromonas hydrophila, Acinetobacter baumannii* and *Comamonas testosteroni*) was prepared using the method of entrapment into phosphorylated polyvinyl alcohol (PVA) beads [7]. Baker's yeast invertase was immobilized on PVA-alginate matrix using boric acid as a cross linking agent [8]. Effects of PVA and boric acid concentrations on diffusion coefficient were investigated using two-level full factorial design. Response surface methodology was used to find the optimum conditions such as pH, temperature and operation stability for immobilizing polygalacturonase on calcium-alginate microspheres [9]. Pectinase enzyme was immobilized on epoxy-activated polymer (DILBEAD-VWR) which was further cross linked with dextran aldehyde. Immobilized enzymes are repeatedly used for the apple juice clarification [10]. Pectinase enzymes were immobilized using calcium-alginate method and process parameters were calculated using 2^4 full-factorial central composite design. Furthermore, thermo-dynamic studies were carried out to estimate efficiency of the immobilized beads [11]. Thermodynamic and kinetic studies on pectinase enzymes from *Aspergillus aculeatus* extractions were carried out and immobilization yield was reported more than 50% [12].

In most studies, resistance to external mass transfer is assumed negligible due to the efficient mixing of the solutions. However, it is not clearly known if the effects due to internal-mass transfer and partition can be eliminated [7]. The applicability of immobilized enzymes in bioprocess industries is based on the efficiency of immobilization, productivity and operational stability. Immobilized pectinase systems are widely used in clarification of fruit juices; hence, this method was used in the present study using guava (*Psidium guajava L.*) fruit pulps. Immobilization of enzymes using entrapment in calcium-alginate is the most popular method for the degradation of pectin since this method is inexpensive and nontoxic for the enzymes. The present study assessed the effectiveness of immobilized enzymes in liquefaction of guava tropical fruit pulps. Novelty of the study was that the fruit pulps were used to determine enzymatic reaction kinetics for soluble and immobilized forms of the enzymes. In general, biochemical engineering studies on effects of diffusion in liquefaction processes are limited. The current study revealed effects of limitations of mass transfer on enzyme kinetics based on parameters such as the particle size and flow rate. Moreover, biodegradation of pectin was studied using stirred batch system and various sized beads of calcium-alginate immobilized pectinase. The average effective diffusivity coefficient was determined using experimental data from calcium-alginate immobilized pectinase.

2. Model description

When enzymes are immobilized in polymer matrices such as calcium-alginate beads, the substrates diffuse into the gel beads and subsequently into the enzymes where the reaction occurs. Diffusion and reaction occur simultaneously. The overall biodegradation rate depends on diffusivity of the substrates within the microporous beads [6]. Making certain assumptions that the beads are spherical, enzymes are uniformly distributed and external mass transfer limitations are negligible and based on Fick's

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Law of Diffusion, an equation is written where the kinetics is represented by Michaelis-Menten equation [4].

$$D_e\left(\frac{d^2C_s}{dr^2}+\frac{2}{r}\frac{dC_s}{dr}\right)-\frac{v_{max}C_s}{K_m+\boldsymbol{C_s}}=0 \quad (1)$$

Where, D_e is the effective diffusivity, r is the radius at a point inside the immobilized beads from the center, C_s is the concentration of substrates, K_m is the Michaelis-Menten constant and v_{max} is the maximum reaction rate. Boundary conditions include r = 0, $\frac{dC_s}{dr}=0$ at $r = R_i$, $C_s = C_{sb}$. Since Equation (1) is a non-linear differential equation, it cannot be evaluated analytically. Various methods are used to solve this problem, but the calculations are difficult and time consuming. Results were presented in the most general and compact form. This was achieved by transforming the highlighted equation into an equivalent dimensionless form [13]:

$$\frac{d^2C_s}{dr^2}+\frac{2}{r}\frac{dC_s}{dr}-9\phi^2\frac{C_s}{1+\boldsymbol{\beta C_s}}=0 \quad (2)$$

Where, ϕ and β dimensionless parameters are defined by the equation.

$$\phi=\frac{Ri}{3}\sqrt{\frac{v_{max}}{D_eK_m}} \quad (3)$$

$$\beta=\frac{C_{sb}}{K_m} \quad (4)$$

When ϕ (Thiele modulus) is sufficiently large, the substrate diffusion is slow compared to when the substrate consumption indicating the rate of reaction is fast. It can be assumed that all the substrate is used within the particle in a thin region adjacent to its external surface; therefore, effects of curvature can be neglected in Equation (2). This results in:

$$D_e\left(\frac{d^2C_s}{dr^2}+\frac{2}{r}\frac{dC_s}{dr}\right)-\frac{v_{max}C_s}{K_m+\boldsymbol{C_s}}=0 \quad (5)$$

$$\text{But}\left(\frac{d^2C_s}{dr^2}\right)=\frac{1}{2}\frac{d}{dC_s}\left(\frac{dC_s}{dr}\right)^2 \quad (6)$$

Equation (6) is substituted in Equation (5) and integrated to get the resulting equation in terms of C_s:

$$\left(\frac{dC_s}{dr}\right)_{r=Ri}=\left[\frac{2}{D_e}\int_{C_{s0}}^{C_{sb}}\frac{v_{max}C_s}{K_m+\boldsymbol{C_s}}dC_s\right]^{1/2} \quad (7)$$

Where, C_{s0} and C_{sb} are the substrate concentrations at the particle center (r = 0) and external surface ($r = R_i$), respectively. In diffusion limited cases, the rate of reaction is fast and hence the concentration at the center becomes zero ($C_{s0} \approx 0$); hence, the integral can be evaluated. The effectiveness factor (η) is defined as [14]:

$$\eta=\frac{Actual\ mean\ reactionrate\ within\ pore}{Rateifnotslowedby\ pore\ diffusion}=\frac{\frac{Ap}{Vp}\left(D_e\left(\frac{dC_s}{dr}\right)_{r=Ri}\right)}{\frac{V_{max}C_{sb}}{K_m+\boldsymbol{C_{sb}}}} \quad (8)$$

Combining equations (7) and (8) and introducing dimensionless quantities will result in:

$$(\eta)_{\phi\gg1}=\frac{1}{\phi}\frac{(1+\beta)}{\beta}\sqrt{2}[\beta-\ln(1+\beta)]^{1/2} \quad (9)$$

Equation (9) provides the effectiveness factor for the case of the reaction mechanism that follows Michaelis-Menten kinetics for the large values of ϕ which indicates that the reaction is diffusion limited. Therefore, large values of ϕ stand for overall reaction rate under diffusion limited conditions.

3. Materials and methods

3.1. Materials

3.1.1. Enzyme source

Pectinase CCM (EC 3.2.1.15) was purchased from Novozymes South Asia (Bangalore, India) and stored at 4°C until use. Activity of the pectinase was estimated according to a method by Kertesz [15]. The polygalacturonase activity was reported as 2,800 U ml^{-1}.

3.1.2. Reagents

Pectin (methoxy content of approximately 6.0% and galacturonic acid content of approximately 65%, 150 grade) and other chemicals were of analytical grades (Akshaya Chemicals, Hyderabad, India). Guava was provided by local gardens (Warangal, India).

3.2. Methods

3.2.1. Immobilization procedure

Method for immobilization of pectinase was selected from other studies [16]. Briefly, 5 ml of 4% (w v^{-1}) sodium-alginate solution (40°C) where mixed with 3.5%, v v^{-1} of pectinase in 5 ml of citrate phosphate buffer (pH 4.5). Then, 0.1 ml of glutaraldehyde solution (40°C) was added to the mixture and gently mixed for 30 min (1st cross linking time of the reaction). This solution was added drop wise into a solution of 0.2 M $CaCl_2$ (40°C) using syringe to form beads of 2.0-2.5 mm. These beads were transferred to a fresh solution of 0.2 M $CaCl_2$ and incubated at 40°C for 1 h. The calcium-alginate beads were decanted and immersed in a solution of glutaraldehyde (0.02% v v^{-1}) for further cross linking (2nd cross linking time of the reaction) at 40°C for 2 h. After immobilization, the alginate beads were washed with distilled water and then with buffer solution to remove free enzymes.

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3.2.2. Assessment of pectin contents in guava fruit pulps

Pectin quantities in the fruit pulps were estimated as calcium-pectate using an original method described by Ranganna for the estimation of pectic substances [17]. Viscosity of the pulp was assessed using Brookfield viscometer. The original pulp was diluted to various concentrations, and viscosities were assessed. The procedure was repeated at various temperatures (10-55°C). Viscosities against pectin contents in the form of percent of calcium pectate can be used as a standard curve for the calibration to assess the concentration of pectin at various viscosities (Fig. 1).

3.2.3. Kinetic studies

The pectin degradation rates using pectinase enzymes are assumed to follow Michaelis-Menten equation in batch systems given by the following equation of:

$$v = \frac{v_{max}\, C_S}{K_m + C_s} \qquad (10)$$

The reaction kinetics were determined using Michaelis-Menten equation and the Michaelis-Menten kinetic parameters (v_{max} and K_m) were assessed during the degradation of pulps using immobilized beads. These included 3.5% (v v^{-1}) of the enzyme in 100 ml of guava fruit pulps containing various initial pectin concentrations (1.82-2.92%, w v^{-1}) at 25°C. Viscosities were assessed with respect to time, and viscosity data were converted to pectin concentrations using the calibration curve.

3.2.4. Assessment of intra-particle resistance due to the mass transfer

To assess the intra-particle resistance due to mass transfer, liquefaction of guava fruit pulps was carried out using immobilized beads that contained enzyme concentrations of 3.5% v v^{-1}. Experiments were repeated at various sizes of immobilized beads (1.93.4 mm). Viscosity data were recorded with respect to time and converted to pectin concentrations using the calibration curve. Since the experiments were carried out in a well-stirred batch reactor, the external resistance due to the mass transfer could be assumed negligible. However, the mass transfer resistance was due to the internal mass transfer resistance only. All the experiments were carried out in duplicate and the arithmetic average values of the data were considered for the calculations.

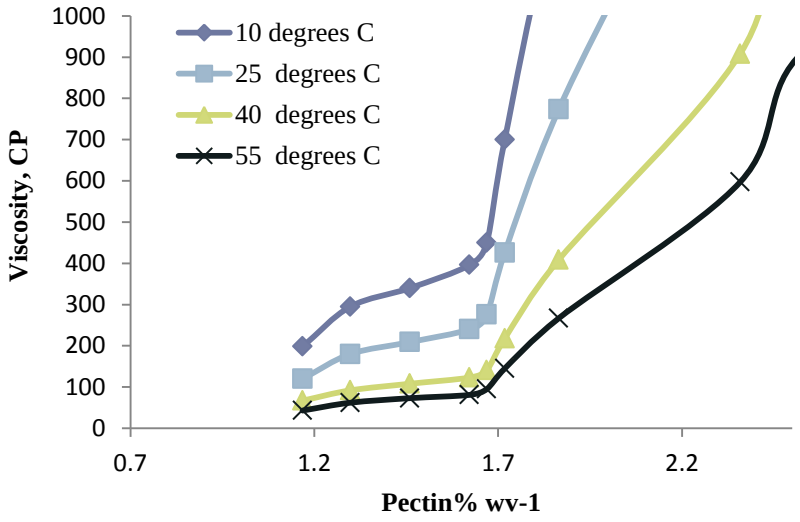

Figure 1. The calibration curves: viscosities against pectin concentrations of guava fruit pulps at various temperatures

4. Results and discussion

4.1. Enzyme Kinetics

Enzyme kinetics for the degradation of pectin in guava pulps were determined based on the pectin concentrations at various time intervals (Fig. 2). In the stated studies, the enzyme concentration was fixed at 3.5% (v v^{-1}) in immobilized beads and the initial concentration of the substrate was changed. Slopes were calculated based on the initial time period from the graphs to find the initial rate of reaction v_0 (Table 1). Graphs were subsequently plotted between v_0 and the corresponding initial substrate concentration. Data were fit well, showing that the reaction followed Michaelis-Menten kinetics [13]. Lineweaver-Burk plot was drawn for $1/v_0$ against $1/C_S$ (Fig. 3). Data were fit with the coefficient of correlation, R^2 = 0.967.

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Then, slope and intercept of the plot were calculated as follows:

Slope = $K_m\ v_{max}^{-1}$ = 2541 sec; intercept = v_{max}^{-1} = 909 sec/pectin % (w v^{-1}).

From which, v_{max} = 0.0011 pectin % (w v^{-1} sec^{-1}) and K_m = 2.795 pectin % (w v^{-1}).

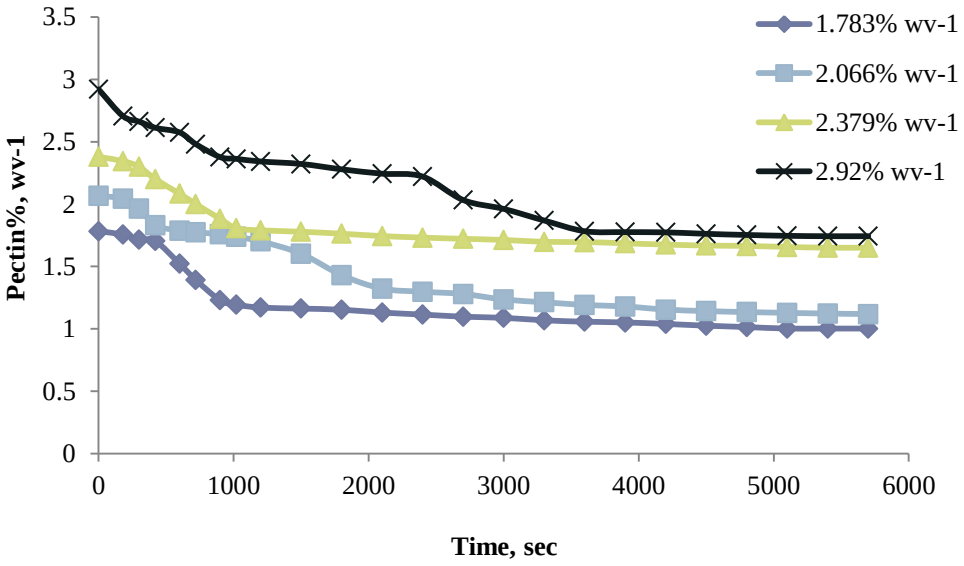

Figure 2. Effects of initial concentrations of pectin on degradation of guava fruit pulps using immobilized beads with 3.5% (v v^{-1}) of pectinase CCM

Table 1. Initial substrate concentrations against initial reaction rates

No.	Substrate conc., C_s, pectin % (w v^{-1})	Initial reaction rate, v_0, pectin% (w $v^{-1}sec^{-1}$) × 10^4	C_s^{-1}	v_0^{-1}
1	1.783	4.33	0.561	2307.6
2	2.066	4.65	0.484	2150.4
3	2.379	4.92	0.420	2033.4
4	2.920	5.01	0.343	1996.4

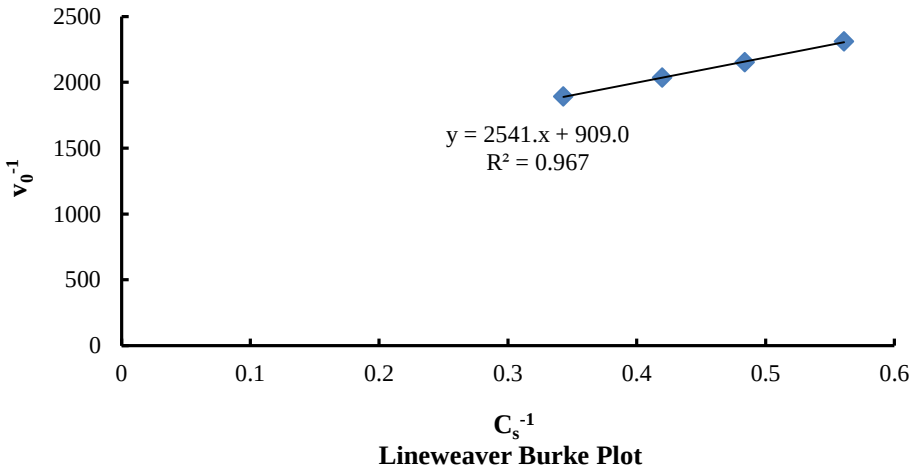

Figure 3. Lineweaver-Burk plot for the immobilized enzymes in guava fruit pulps

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Table 2. Data of η, Φ and D_e for various sized immobilized beads*

S No.	D_p, mm	Initial degradation rate, v_o pectin %, ($wv^{-1}sec^{-1}$) × 10^3	η	Φ	$D_e \times 10^{12}$, $m^2\ sec^{-1}$
1	Free enzyme	1.219	1	---	---
2	1.9	0.634	0.5201	3.0546	4.2297
3	2.5	0.475	0.3897	4.0768	4.111
4	3.4	0.327	0.2683	5.9214	3.604

*Pectinase enzyme concentration of 3.5% (v v^{-1}); initial pectinase concentration of 2.92% (w v^{-1})

4.2. Effects of mass transfer resistance

Pectin degradation rate was assessed from the initial slope of pectin concentration against time graph using 3.5% (v v^{-1}) pectinase enzyme in immobilized beads (for the free and various sized beads) (Fig. 4) for an initial pectin concentration of 2.92% (w v^{-1}). From the plot, it could be seen that the pectin degradation for free enzymes was higher and the biodegradation rate became slow as the size of the bead particles increased. Immobilized enzymes showed a slower pectin degradation rate than that soluble enzymes did. It is noteworthy that immobilized enzymes can be reused and immobilization protects the enzyme molecules, which are more stable to changes at various temperatures and pH. Rates were calculated using an initial slope method (Fig. 4) and tabulated in Table 2. The effectiveness factors were determined experimentally by calculating the ratio of degradation rate with mass transfer limitation for immobilized enzymes to degradation rate with no mass transfer limitation for soluble enzymes [6]. The effectiveness factors respectively included 0.52 and 0.2683 for the smallest and largest bead sizes, which are less than 1. Therefore, it could be concluded that the degradation rate was diffusion limited. The V_{max} and K_m were calculated from the kinetic data of immobilized enzymes as follows:

v_{max} = 0.0011 pectin % w v^{-1} sec^{-1}; K_m = 2.795 pectin% w v^{-1}; C_{sb} = 2.92 pectin% w v^{-1}

The ϕ Thiele modulus was calculated using Equation 9 and the effective diffusivity using Equation 3) (Table 2). The ϕ values were greater than 1, which showed that intra-particle mass transfer resistance was limiting the overall rate of biodegradation [14]. Beads with lower diameters resulted in higher D_e and hence, indicating a better rate of substrate diffusion to the active sites from the bulk solution. The decrease in D_e as the bead size increased may be due to the reduction in the volume available for the substrate to move in and increase in the path length for movement of substrate.

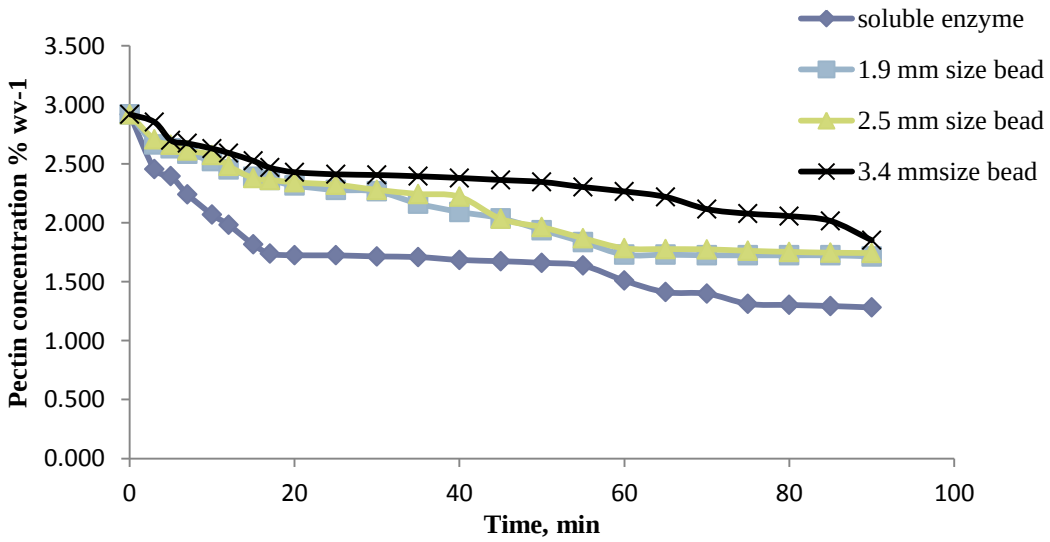

Figure 4. Effect of bead size on pectin degradation in guava fruit pulp

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5. Conclusion

Liquefaction of fruit pulps for the production of clarified fruit juices is an established industrial process using pectinase enzymes. These enzymes can be used in suspension or immobilized forms. In this study, pectin degradation was investigated in a batch reactor system using free and immobilized pectinase enzymes. Based on the effectiveness factors, it is concluded that the diffusion resistance is significant and must not be neglected in the analysis. Increased values of Φ with increased sizes of beads have shown that the substrate concentration drops quickly on moving into the pores. In contrast, mass transfer strongly influences the progress of biochemical reactions (degradation of pectin). Mass transfer resistance can be eliminated with higher turbulences, higher substrate concentrations and smaller particle sizes.

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6. Conflict of interest

The authors declare no conflict of interest.

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تاثیر ضریب نفوذپذیری تجزیه پکتین در پالپ گوآوا (*پسیدیوم گواجاوا ال.*) با استفاده از پکتیناز تثبیت شده

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چکیده

سابقه و هدف: اخیرا نشان داده شده است که استفاده از فناوری تثبیت از طریق به دام اندازی سلول به منظور ثبات زیست کاتالیزورها روشی اقتصادی می باشد زیرا مزایایی در برابر کشت‌های غوطه ور دارد مانند قابلیت استفاده مجدد، غلظت بالای سلول در یک دوره زمانی، حذف فرایند هزینه‌بر بازیابی و بازیافت سلول و ساده کردن فرایند پایین دست. آگاهی از ضرایب انتشار و نفوذپذیری برای بهینه‌سازی ریزپوشانی ضرورت دارد. اهداف اصلی مطالعه حاضر مطالعات تجزیه پکتین با استفاده از سامانه ناپیوسته‌ای حاوی آنزیم‌های پکتیناز محلول و تثبیت شده است.

مواد و روش‌ها: برای به صورت مایع درآوردن پالپ گوآوا *(پسیدیوم گواجاوا ال.)* که کاربرد گسترده‌ای در صنایع غذایی دارد، پکتیناز تجارتی به روش به دام اندازی تثبیت شد. پالپ میوه گوآوا با استفاده از آنزیم‌های پکتیناز آزاد و تثبیت شده به صورت مایع درآورده شد تا مقاومت در برابر انتقال جرم درون ذره‌ای تعیین شود.

یافته‌ها و نتیجه‌گیری: در مطالعه حاضر، تاثیر انتقال جرم بر فرایند مایع‌سازی نشان داده شد. عوامل موثر بر آن برای اندازه‌های گوناگون دانه‌های تثبیت شده کمتر از ۱/۰ بود که نشان می‌دهد تجزیه پکتین فرایندی با انتشار کنترل شده بوده است. عوامل موثر شامل ۰/۵۲۰ و ۰/۲۶۸ به ترتیب برای کوچکترین و بزرگترین قطر دانه‌ها بود. مقاومت در برابر انتقال جرم درون ذره‌ای با محاسبه مدول تیل (ϕ) با استفاده از عوامل موثر محاسبه شده برای دانه-ها با اندازه‌های گوناگون به دست آمد. این نتایج بعدا برای محاسبه ضرایب انتشار موثر (D_e) پالپ گوآوا به حفرات مورد استفاده قرار گرفتند. اعداد مدول تیل (ϕ) بسیار بیشتر از ۱ بودند که نشان می‌دهد واکنش بسیار سریع روی داده و در سامانه انتقال جرم کنترل شده بوده است. دانه‌هایی با قطر کمتر و ضرایب انتشار بالاتر، نرخ انتشار بهتر رشدمایه[1] از محلول حجیم را از خود نشان دادند.

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***نویسنده مسئول**

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[1] Substrate

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